The Role of JAK2 in Myeloproliferative Neoplasms

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Abstract

Myeloproliferative Neoplasms (MPNs) are a group of clonally derived stem-cell disorders of haematopoietic progenitors resulting in a proliferation of differentiated myeloid cell types. They include polycythaemia vera (PV), essential thrombocythaemia (ET), and myelofibrosis (MF). JAK2 encodes a non-receptor tyrosine kinase and mutations in this gene are found in a large percentage of MPN cases. It is present in approximately 95% of PV and between 50-60% of ET and PMF cases. JAK2 plays a key role in cell proliferation and differentiation of haematopoietic stem cells to mature blood cells.

Expression of key haematopoietic genes were studied along with their relationship to haematological parameters and JAK2 mutational status. Anagrelide was used to target the haematopoietic transcription factor, GATA1, in MPN model cell lines. The effect of this drug on downstream effectors of megakaryocytic differentiation was also studied to determine potential mechanisms for its anti-platelet activity in essential thrombocythaemia. Ruxolitinib, a JAK1/2 inhibitor, was used to block JAK2 and the downstream effects on STAT, SOCS and interferon-gamma target proteins were quantified. Global proteomic changes were studied, using isobaric tagging and relative quantification and LC-MS/MS. This inhibitor was also used to examine the importance of JAK2 signalling in erythropoiesis and colony growth in primitive progenitor cells isolated from MPN patients.

GATA1 expression was found to be dysregulated in the PBMCs of ET patients. In cell lines, expression of downstream GATA1 targets were found to be downregulated during inhibition of megakaryopoiesis using anagrelide. Functional protein analysis showed that STAT1 and associated key molecular pathways involved in interferon signalling were the target of JAK2 inhibition by ruxolitinib.

The results in this study suggest a potential role for GATA1 as a disease biomarker, independent of JAK2 mutational status. The activity of the anti-platelet drug, anagrelide, may in part be due to targeting of GATA1 downstream targets. STAT1 is likely to play a key role in MPN pathogenesis and response to JAK inhibitor therapies.

Contents

Abbreviations:

- AML: Acute myeloid leukaemia
- AMKL: Acute megakaryoblastic myeloid leukaemia
- ANA: Anagrelide
- ATP: Adenosine triphosphate
- BFU-E: Burst forming unit - erythroid
- BM: Bone marrow
- BSA: Bovine Serum Albumin
- CALR: Calreticulin
- cAMP: Cyclic adenosine 3, 5-monophosphate
- CANX: Calnexin
- CDK: Cyclin dependent kinase
- cDNA: Complimentary DNA
- CEL/HES: Chronic eosinophilic leukaemia / hypereosinophilic syndrome
- CFC: Colony forming cell
- CFU-E: Colony forming unit – erythrocyte
- CFU-GEMM: Colony forming unit – granulocyte, erythrocyte, monocyte, megakaryocyte
- CFU-GM: Colony forming unit – granulocyte, monocyte
- cGMP: Cyclic guanosine 3, 5-monophosphate
- CML: Chronic myeloid/myelogenous leukaemia
- CNL: Chronic neutrophilic leukaemia
- DMSO: Dimethyl sulfoxide
- EPO Erythropoietin
- ER: Endoplasmic reticulum
- ET: Essential thrombocythaemia
- FBS: Foetal Bovine Serum
- FOG1: Friend of GATA1
- GM-CSF: Granulocyte colony stimulating factor
- GO: Gene ontology
- GPIX: Glycoprotein 9
- HDAC: Histone deacetylase
- HL: Hodgkin's lymphoma
- HSC: Haematopoietic stem cell
- HSP: Heat shock protein
- HU: Hydroxyurea/hydroxycarbamide
- IC_{50}: Half maximal inhibitory concentration
- IFN: Interferon
- IL: Interleukin

- IRF: Interferon receptor factor
- iTRAQ: Isobaric tagging and relative quantification
- JAK: Janus kinase
- KIR: Kinase inhibitory region
- LC-MS/MS: Liquid chromatography with tandem mass spectroscopy
- mAb: Monoclonal antibody
- MCD: Mast cell disease
- MDS: Myelodysplastic syndrome
- MF: Myelofibrosis
- MPD: Myeloproliferative disorder
- MPN: Myeloproliferative neoplasm
- mRIPA buffer: Modified radioimmunoprecipitation buffer
- mRNA: Messenger RNA
- NFE2: Nuclear factor erythroid 2
- PAGE: Polyacrylamide gel electrophoresis
- PBMC: Peripheral blood mononuclear cell
- PBS: Phosphate buffered saline
- PDE3: Phosphodiesterase type III
- PF4: Platelet factor 4
- PSTPIP2: Proline-serine-threonine phosphatase interacting protein 2
- PV: Polycythaemia vera
- qPCR: Quantitative polymerase chain reaction
- rhEPO: Recombinant human erythropoietin
- RUX: Ruxolitinib
- SDS: Sodium dodecyl sulphate
- SOCS: Suppressors of cytokine signalling protein
- STAT: Signal transducers and activators of transcription
- TMD: Transient myeloproliferative disorder
- TF: Transcription factor
- TK: Tyrosine kinase
- TPO: Thrombopoietin
- TBS: Tris buffered saline
- PMA: Phorbol 12-myristate 13-acetate
- WHO: World Health Organisation

CHAPTER 1: Introduction

1.1: Background

Myeloproliferative neoplasms (MPN) are a group of bone marrow diseases arising from clonal disorders of haematopoietic stem cells (Nangalia *et al.*, 2016). They manifest in the overproduction of one or more types of mature blood cell. Traditionally, they have been classified into one of two groups, Philadelphia chromosome positive (Ph1+) or negative (Ph1-), depending on the presence or absence of a chromosomal translocation (t(9;22) (q34;q11)), resulting in the expression of a constitutively active fusion gene product, breakpoint cluster region-Abelson tyrosine kinase 1(BCR-ABL1).

1.2: BCR-ABL1

A chromosomal abnormality present in patients with chronic myeloid leukaemia (CML), but not in those with acute myeloid leukaemia (AML), was identified in 1960 (Hungerford and Nowell, 1960). Rowley (1973) determined that this abnormality was the result of a reciprocal translocation between chromosome 9 and 22. The ABL1 gene on chromosome 9 is fused with the breakpoint cluster region (BCR) gene to generate a fusion gene *BCR-ABL1*. The resulting chimeric BCR-ABL1 protein that is expressed is a constitutively active tyrosine kinase. There are three main variants of the BCR-ABL1 protein, p190, p210 and p230, which are each associated with a particular disease phenotype and kinase activity, with p210 the subtype found primarily in CML (Li *et al.*, 1999).

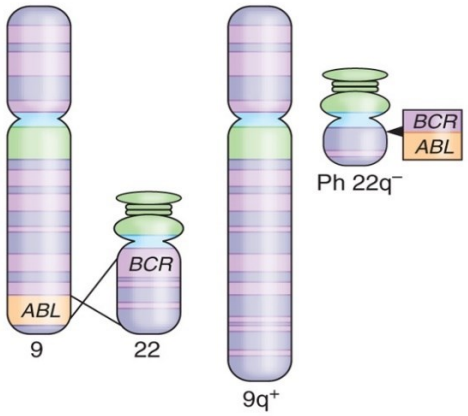


Figure 1: Diagram of reciprocal translocation in CML and the generated Philadelphia Chromosome (Lydon, 2009).

1.3: The BCR-ABL negative MPN

"Myeloproliferative disorders" were first described as a distinct group by Dameshek in the 1950s to describe several disorders that he speculated shared a common origin (Dameshek, 1951, Tefferi, 2008). Essential thrombocythaemia (ET), polycythaemia vera (PV) and myelofibrosis (MF) had all been identified by the early twentieth century, and trilineage myeloproliferation had been recognised in PV and other MPDs (Dameshek, 1951, Tefferi, 2008). These were formally classified CML, PV, ET and MF along with erythroleukaemia (later redefined as acute erythroid leukaemia forming a subtype of AML) (Dameshek, 1951). Dameshek recognised that despite these disorders being primarily associated with one cell type, it often coincided with proliferation of others. Further to this, he proposed that the various manifestations of disease occurred as a result of differential stimulatory activity on bone marrow cells. At the time, the source of the myelostimulatory activity was speculated to be endogenous steroid hormones (Dameshek, 1951).

1.4: Myeloproliferative Neoplasms: WHO Classification (2001 – 2016)

In 2001, the World Health Organisation attempted to classify the numerous myeloproliferative disorders resulting in four groupings, 1) chronic myeloproliferative disorders (CMPD), 2)

myelodysplastic syndromes (MDS), 3) MDS/MPD crossover diseases and 4) mast cell disease (MCD). CMPD included PV, ET, MF, CML, as well the rarer chronic neutrophilic leukaemia (CNL), chronic eosinophilic leukaemia (and the hypereosinophilic syndrome) (CEL/HES) and CMPD unclassified. The principal criteria for classification in the CMPD group was a shared effective clonal myeloproliferation (Tefferi and Vardiman, 2008).

The discovery of molecular disease markers, including the *JAK2*^{V617F} somatic mutation in 2005, led to the revised 2008 WHO classification, with myeloproliferative diseases renamed myeloproliferative neoplasms (MPNs) (Tefferi and Vardiman, 2008). The major changes to the 2001 classification included a separate grouping outside of MPN for myeloid neoplasms associated with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* and *FGFR1* (Bain, 2010). MCD was grouped with the MPNs, in recognition that like other MPNs it arises from a clonal stem cell disorder (Tefferi and Vardiman, 2008).

Diagnostic criteria for the MPNs were also further defined in the 2008 WHO classification, the aforementioned *JAK2*^{V617F} mutation providing additional information to assist with diagnosis (Vardiman *et al.*, 2009). The *JAK2*^{V617F} mutation is not in itself specific to any of the MPNs but can be used along with other diagnostic criteria to eliminate other causes (Bench *et al.*, 2013).

Although the 2008 diagnostic criteria and associated algorithms appear to provide a mechanism for clearly distinguishing between different MPNs, there remained debate concerning patients whose symptoms overlap more than one of these conditions, in particular whether patients diagnosed with ET should be categorised as having either “true ET” or “pre-fibrotic MF”. Campbell *et al.* (2009a) demonstrated that the reticulin grade at diagnosis was a prognostic marker for disease activity and duration. However, these findings were disputed by Thiele (2009), who questioned whether a significant cohort of these patients should have been classified as MF with thrombocythaemia rather than “true” ET. Counter to this, the Campbell group argued that 17% of their patients did not meet the strict WHO guidelines for either ET or MF diagnosis, but clearly had a myeloproliferative neoplasm (Campbell *et al.*, 2009b). The major issue of contention between the groups would appear to concern

whether narrow diagnostic criteria, such as that for prefibrotic MF have a clinical utility over broad categories that aim to capture all patients. Barosi *et al.* (2012) demonstrated that prefibrotic MF was aligned in a biological and clinical continuum with myelofibrosis. It has previously been proposed that all the classic Ph1- MPNs form a continuum, where progression from a chronic to an accelerated chronic and finally acute phase of disease is represented by PV and ET developing to MF and finally AML (Campbell *et al.*, 2005).

In an attempt to address the issues surrounding ET and MF diagnoses, the WHO have released updated criteria distinguishing pre-MF from "true" ET (Arber *et al.*, 2016). The diagnostic criteria for ET, pre-MF and overt MF have now also been updated to recognise the CALR mutation (Klampfl *et al.*, 2013, Nangalia *et al.*, 2013). Guidance has also been provided to myelofibrosis grading, which is used in distinguishing between the ET, pre-MF, and overt-MF disease continuum.

Potential under-diagnosis of PV has also been addressed in the 2016 revision to MPN classification of myeloid neoplasms. A study of patients with the *JAK2*^{V617F} mutation and bone marrow morphology consistent with the 2008 WHO criteria but haemoglobin levels lower than the major diagnostic criteria showed significantly increased risk of disease transformation to MF and acute leukaemia compared to diagnosed PV patients (Barbui *et al.*, 2014). It is not yet known whether this is an early form of PV or whether it represents an initial myelofibrosis stage given its poorer prognosis (Barbui *et al.*, 2014). For now the diagnostic criteria for haemoglobin levels have been reduced and greater importance is attached to bone marrow morphology (now a major criteria) meaning these cases are grouped with PV (Arber *et al.*, 2016). A typical flow chart for the steps involved in MPN diagnosis is illustrated in Figure 2**.**

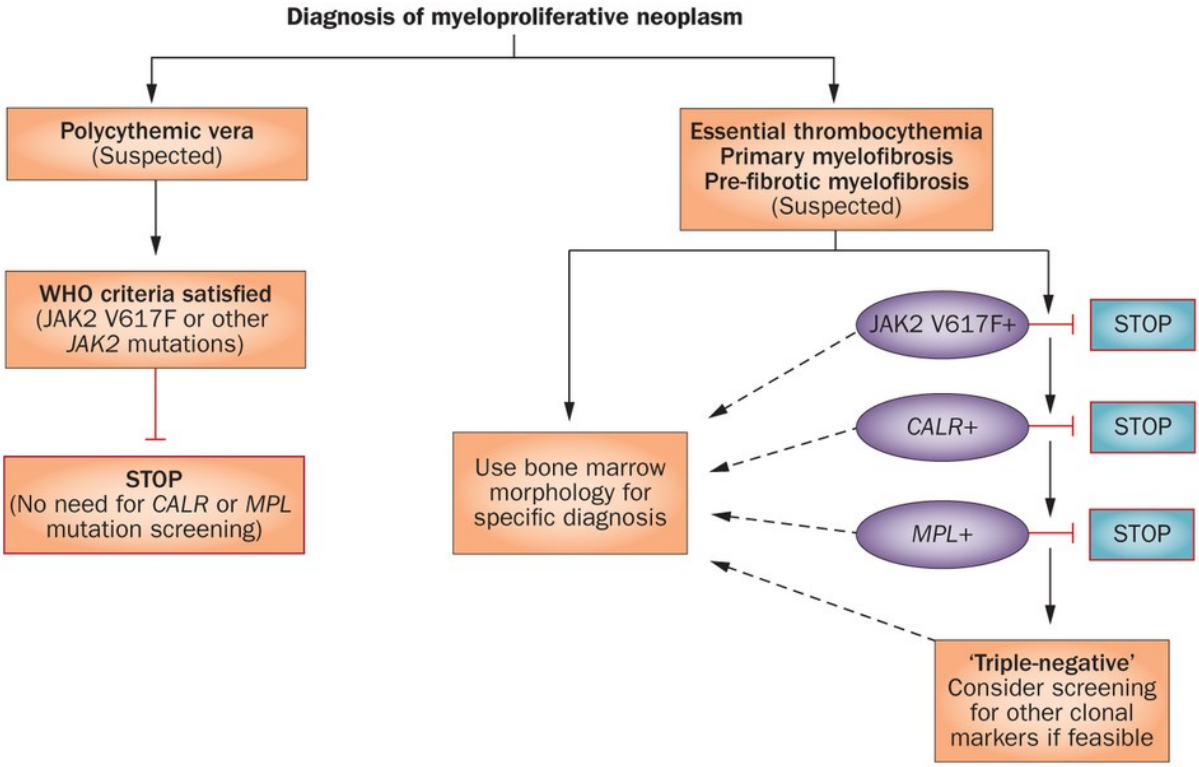


Figure 2: Typical diagnostic algorithm for MPNs (Tefferi and Pardanani, 2014).

1.4.1: Polycythaemia Vera (PV)

PV is a myeloproliferative neoplasm characterised by an elevated number of erythrocytes, greater than 25% red cell mass above normal (Arber *et al.*, 2016). This overproduction of red blood cells can lead to vascular and thrombotic complications which can have serious implications for the patient's quality of life (Stein *et al.*, 2014). Some of the symptoms commonly encountered in PV include pruritus, fatigue, night sweats, bone pain, thrombosis, and bleeding (Griesshammer *et al.*, 2015). Median age of diagnosis of patients is between 55 and 60 years old (Harrison and Keohane, 2013). Annual incidence rate has been estimated at 0.86 per 100,000 based on pooled data from European studies (Titmarsh *et al.*, 2014). The risk for transformation to myelofibrosis (MF) and acute myeloid leukaemia over 10-15 years is 10-15% and 5-10% respectively (Harrison and Keohane, 2013).

Table 1: 2016 WHO Diagnostic Criteria for PV. A diagnosis of PV is determined by the presence of all three major criteria or the 1st two major criteria and the minor criteria (Arber *et al.*, 2016).

		POLYCYTHAEMIA VERA
Major Criteria	1	Haemoglobin > 165/160 g/L (men/women) OR Haemocrit > 49/48% (men/women) OR Elevated red cell mass > 25% above mean normal predicted value
	2	BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size). This criterion may not be required in cases with sustained absolute erythrocytosis, haemoglobin levels > 185/165 g/L and haematocrit > 49.5%
	3	Presence of *JAK2*^{V617F} or similar mutation
Minor Criteria	1	Subnormal serum EPO level

1.4.2: Essential Thrombocythaemia (ET)

ET is defined by an elevated circulating platelet count (> 450 x 10^9 /L) and increased numbers of megakaryocytes (Tefferi and Vardiman, 2008). Diagnosis also requires elimination of other myeloproliferative disorders. The presence of a clonal marker such as *JAK2*^{V617F} is indicative, as well as

ruling out secondary or reactive thrombocytosis as a cause of elevated platelet counts. Overall median survival (19.8 years) is longer than seen in PV (13.5 years) but still lower than the age-matched control population (Tefferi *et al.*, 2014a). Reflecting the longer survival rates, the risk of disease transformation, myeloid or leukaemic, is low in the first decade of life (9.1% and 1.4% respectively) but increases significantly into the second (28.3 and 8.1%), and third decades after diagnosis (58.5% and 24.0%) (Wolanskyj *et al.*, 2006).

Table 2: 2016 WHO Diagnostic Criteria for ET. All four major criteria required for diagnosis or first three major criteria and the minor criteria (Arber *et al.*, 2016).

		ESSENTIAL THROMBOCYTHAEMIA
Major Criteria	1	Platelet count ≥ 450 x 10^9 /L
	2	BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibres
	3	Not meeting WHO criteria for CML, PV, MF, MDS or other myeloid neoplasm
	4	Presence of *JAK2*, *CALR*, or *MPL* mutation
Minor Criteria	1	Presence of a clonal marker or absence of evidence for reactive thrombocytosis

Table 3: 2016 WHO Diagnostic Criteria for pre-MF. Diagnosis requires meeting all three major and one minor criteria. Minor criteria to be confirmed twice on consecutive investigations (Arber *et al.*, 2016).

		PRE-MF
Major Criteria	1	Megakaryocytic proliferation and atypia, without reticulin fibrosis > grade 1, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis
	2	Not meeting WHO criteria for CML, ET, PV, MF, MDS, or other myeloid neoplasm
	3	Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker, or absence of reactive myelofibrosis
Minor Criteria	1	Anaemia not attributed to a comorbid condition
	2	Leucocytosis: ≥ 11×10^9/L
	3	LDH increased to above upper normal limit of institutional reference range
	4	Palpable splenomegaly

1.4.3: Myelofibrosis (MF)

Myelofibrosis is the least common of the three main non-CML myeloproliferative neoplasms, accounting for approximately 0.47 new cases per 100,000 (Titmarsh *et al.*, 2014). It is nonetheless the most serious and has a worse prognostic outcome than either PV or ET (Tefferi *et al.*, 2014a). Median survival in MF is 5.9 years (Tefferi *et al.*, 2014a). Blast transformation and progression to AML occurs in up to 25% of MF patients (Harrison and Keohane, 2013). Constitutional symptoms have a significant

negative impact on quality of life, these arise as a result of bone marrow failure or splenomegaly and include sweating, pruritus, fever and bone pain (Harrison and Keohane, 2013).

Table 4: 2016 WHO Diagnostic Criteria for overt MF. Diagnosis requires meeting all three major and one minor criteria. Minor criteria to be confirmed twice on consecutive investigations (Arber *et al.*, 2016).

		MYELOFIBROSIS
Major Criteria	1	Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3
	2	Not meeting WHO criteria for CML, ET, PV, MDS, or other myeloid neoplasm
	3	Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker, or absence of reactive myelofibrosis
Minor Criteria	1	Anaemia not attributed to a comorbid condition
	2	Leucocytosis: $\geq 11 \times 10^9$/L
	3	LDH increased to above upper normal limit of institutional reference range
	4	Palpable splenomegaly
	5	Leucoerythroblastosis

1.5: Janus Kinases (JAK)

Janus kinases (JAK) are a family of four cytoplasmic non-receptor tyrosine kinases, comprising of JAK1-3 and TYK3 (Yamaoka *et al.*, 2004). Janus kinase family members, despite their structural similarities, play crucial and non-redundant roles in multifunctional areas such as embryonic development, haematopoiesis, and immune regulation. JAK1, JAK2 and TYK2 are ubiquitously expressed, while JAK3 is found mostly in haematopoietic cells (Ghoreschi *et al.*, 2009). The four JAK kinases share a common structure: receptor-binding domains are located at the N-terminal, while the catalytic active kinase and pseudokinases are at the C-terminal (Degryse *et al.*, 2014). They are responsible for transmitting signals from extracellular stimuli across the cell membrane and through the cytoplasm to the nucleus. Cytokine receptors on the cell surface lack protein kinase domains and rely on the catalytic activity of non-receptor tyrosine kinases, such as JAK2, for phosphorylation and recruitment of other signal transducing proteins (Babon *et al.*, 2014). JAK2, as with other members of the JAK protein family, is comprised of seven homology domains (Figure 3). JH1 is located closest to the carboxyl end and JH7 nearest the N-terminal end. JH1 contains the catalytic activity while JH7 is responsible for receptor binding (Figure 3).

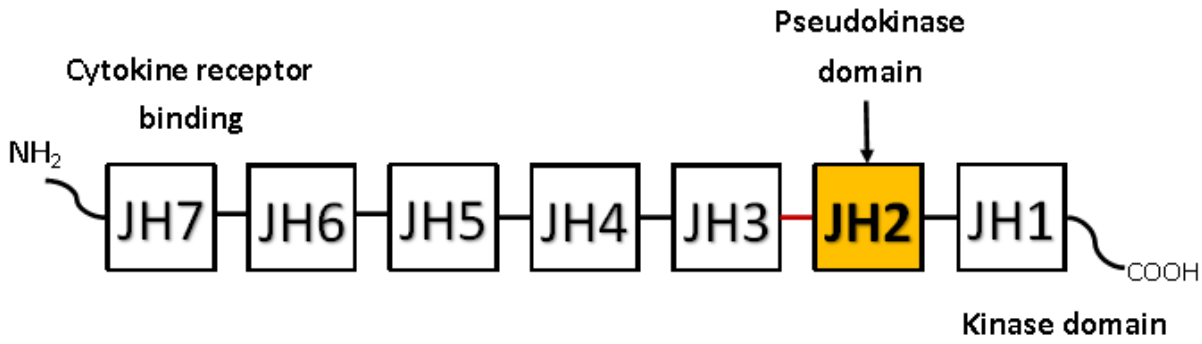


Figure 3*:* Homology domains of JAK2. Receptor binding occurs at the N-terminus while kinase activity (JH1) is at the carboxyl end.

JAK is activated by cytokine binding to the receptor, resulting in dimerization or a conformational change leading to increased kinase activity (Furqan *et al.*, 2013). Activated JAKs can then in turn phosphorylate tyrosine residues on the cytoplasmic portion of the receptor. JH2 shares much homology with JH1 domain but lacks enzymatic activity and is regarded as a pseudokinase domain (Furqan *et al.*, 2013). It has an inhibitory effect on JH1 through the phosphorylation of two negative regulatory sites in JAK2, Ser523 and Tyr570 (Ungureanu *et al.*, 2011).

Experiments have shown that both *JAK1* and *JAK2* knockout result in a lethal phenotype. *JAK1* null mice ($JAK^{-/-}$) are smaller in size and die a few hours after birth (Rodig *et al.*, 1998, Sakamoto *et al.*, 2016). Experiments with cells from *JAK1* knockout mice showed a lack of biologic responses to type II cytokine receptors, including those for interferon-α (IFN-α) and interferon-γ (IFN-γ) (Rodig *et al.*, 1998), and *JAK1* deficient LSK cells do not respond to type 1 interferons (Kleppe *et al.*, 2016). Knockout of *JAK2* in mice produces an embryonic lethal phenotype by 12.5 days; conditional knockout from E12.5 to 4 days post-natal also resulted in death due to impaired haematopoiesis (Park *et al.*, 2013). Deletion of JAK2 in adult mice (> 2 months) resulted in severe impairment of erythropoiesis and thrombopoiesis, and lethality in 20% of cases (Park *et al.*, 2013).

Mutations in *JAK2* are found in a wide range of haematological malignancies. Most frequently, mutations in the pseudokinase domain or in the nearby exon 12 are associated with myeloproliferative neoplasms, PV, ET, and MF (Lundberg *et al.*, 2014). Mutations are also found in some rare cases of *de-novo* acute myeloid leukaemia (AML) (Fröhling *et al.*, 2006, Lee *et al.*, 2006, Steensma *et al.*, 2006). Translocations of the *JAK2* gene locus, resulting in a JAK2 fusion protein have also been reported in cases of AML and CML (Ho *et al.*, 2010). Mutations and translocations in the 9p24 region of the *JAK2* gene have been detected in over 30% of Hodgkin's lymphomas (HLs) (Meier *et al.*, 2009, Van Roosbroeck *et al.*, 2011).

TYK2 knockout in mice, whilst not lethal, results in a partial reduction in response to cytokine signalling from both type I and type II interferons (Karaghiosoff *et al.*, 2000). Severe combined

immunodeficiency results from JAK3 knockout, with a reduction in the number of functional T and B cells (Ward *et al.*, 2000). Myelopoiesis is also adversely affected, JAK3 null mice shows signs of splenomegaly and have increased numbers of neutrophils (Grossman *et al.*, 1999). Mutations in the pseudokinase domain of JAK3 have been identified in patients with acute megakaryoblastic myeloid leukaemia (Walters *et al.*, 2006).

1.6: JAK signalling and STAT

Cytokine signalling through transmembrane receptors and downstream signalling through intracellular pathways are important modulators for cell proliferation, differentiation, and growth. Haematopoietic cytokine receptors are divided into 5 groups (Figure 4) depending on the number of subunits or the presence of shared subunits (Baker *et al.*, 2007).

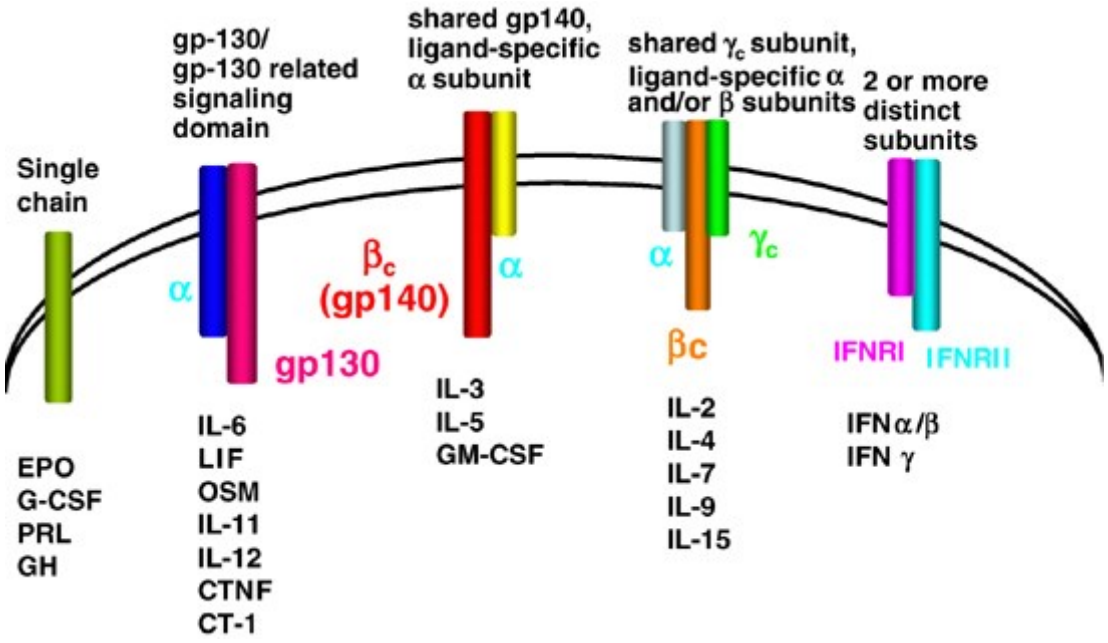


Figure 4: Diagram showing the five main haematopoietic cytokine receptor groups and their associated cytokines (Baker *et al.*, 2007).

Receptors for erythropoietin (EPO), thrombopoietin (TPO), granulocyte colony stimulating factor (G-CSF) as well as some interleukins (IL), and type II interferons are lacking in intrinsic catalytic activity

(Staerk and Constantinescu, 2012), and therefore require additional signalling downstream from the initial cytokine-receptor binding.

As described previously, JAK2 is activated by receptor-ligand binding and dimerization which in turn leads to phosphorylation of tyrosine residues on the cytoplasmic ends of the membrane bound receptor. The confirmation change induced by this phosphorylation allows the binding of SH2 domain containing molecules, including signal transducers and activators of transcription (STATs), src kinases and protein phosphatases. In the case of STAT proteins, a conserved tyrosine residue is located at the C-terminal and this is phosphorylated by JAK2. Phosphorylation of STAT leads to dimerization and translocation to the nucleus, where it acts as a transcription factor (Figure 5).

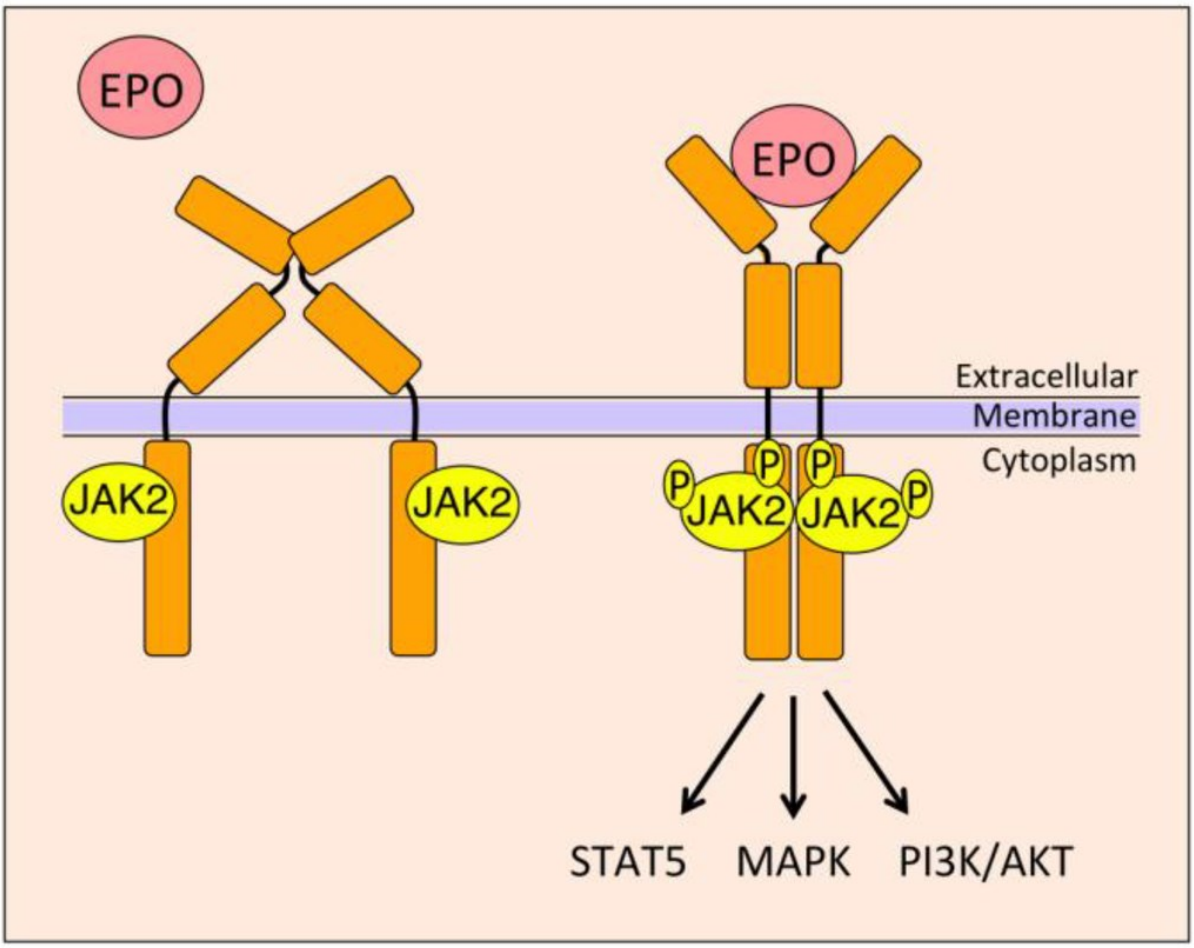


Figure 5: Mechanism of JAK2/STAT5 signalling through erythropoietin binding the EPO receptor (Zhang et al., 2014).

There are seven STAT proteins identified in humans, STAT 1-4, 5a, 5b and 6, ranging in size from 73 to 95 kDa (Steelman *et al.*, 2004). The protein structure of members of this family consists of an N-terminal domain, a central DNA binding domain, an SH2 domain and a transactivation domain located near the C-terminal (Steelman *et al.*, 2004). The N-terminal contains an oligomerization domain which is phosphorylated by JAK activity, this phosphorylation allows interaction with SH2 domains on other STAT proteins and resulting dimerization in the cytoplasm. STAT dimers can then translocate to the nucleus and bind recognised sequences of DNA (Chen *et al.*, 1998), where they act as transcription factors, affecting the expression of one or more genes.

STAT activity is controlled through a number of different mechanisms, both constitutive and inducible. Constitutive suppressors include tyrosine phosphatases, SHP1 and 2 in the cytoplasm and a family of STAT inhibitors called protein inhibitors of activated STATs (PIAS) (Valentino and Pierre, 2006). Suppressors of cytokine signalling proteins (SOCS) are inducible negative regulators of JAK/STAT activity (Tamiya *et al.*, 2011). Members of this family of proteins contain a central src-homology 2 (SH2) domain and a carboxyl terminal end of forty amino acids known as a SOCS box, which is required for proteosomal degradation of SOCS binding partners (Zhang *et al.*, 1999). There are eight members of the SOCS protein family, SOCS1-7 and cytokine-inducible SH2 protein (CIS) (Yoshimura and Yasukawa, 2012). Downregulation of *SOCS1* and inactivating mutations are linked to a wide range of solid tumours, as well as haematological malignancies, including AML, multiple myeloma, B-cell lymphomas and HL (Inagaki-Ohara *et al.*, 2013, Mottok *et al.*, 2009). Two members of the SOCS family (SOCS1 and SOCS3) are known to interact directly with JAK2 (Trengove and Ward, 2013). Both SOCS1 and SOCS3 have a kinase inhibitory region (KIR), located with the N-terminal domain (Tamiya *et al.*, 2011). Adjacent to the KIR, they can also target JAK2 for ubiquitination and degradation by the previously described SOCS box (Hookham *et al.*, 2007). SOCS1, 2 and 3 are unable to control the increased kinase activity of $JAK2^{V617F}$ expressing cells and SOCS3 enhances $JAK2^{V617F}$ induced proliferation (Hookham *et al.*, 2007). Hypermethylation of CpG islands located in the promoter regions of *SOCS1* and *3* genes have been identified in PV and ET patients, including those positive and negative

for the *JAK2*^{V617F} mutation (Teofili *et al.*, 2008). Silencing of genes involved in tumour suppression and cell cycle control is a characteristic of CpG island methylation (Melzner and Möller, 2003).

As well as the STAT pathways, other mechanisms of downstream signalling can be affected by changes to JAK2. JAK2 itself can also translocate to the nucleus and affect chromatin structure through phosphorylation of Tyr41 on histone H3 (Dawson *et al.*, 2009). This prevents binding of H1 alpha to this region. Inhibition of JAK2 results in the down-regulation of a haematopoietic oncogene, *LMO2*, reduction in H3Y41 phosphorylation at the *LMO2* promoter site and an increase in H1 alpha binding at the same site (Dawson *et al.*, 2009).

1.6.1: PI3K/Akt

The PI3K/Akt pathway is a major cytokine signalling axis responsible for many functions in haematopoiesis. Tyrosine kinase receptors in the plasma membrane bind their respective ligands and activate P13K in the cytoplasm. PI3K phosphorylates the 3-OH position on the inositol ring of phosphatidylinositol, generating the second messenger phosphatidylinositol-3,4,5-trisphosphate (Fresno Vara *et al.*, 2004). This indirectly acts upon a serine/threonine kinase, protein kinase B/Akt, attracting it to the cell surface where it can be phosphorylated and activated by phosphoinositide dependent kinases 1 and 2 (Vivanco and Sawyers, 2002). Activated Akt can then work downstream via its pathway to effect the expression of a number of genes affecting proliferation, apoptosis and growth (Vivanco and Sawyers, 2002, Fresno Vara *et al.*, 2004).

Mutations in this pathway, both upstream and downstream have been implicated in a wide range of cancers, but are seen at low frequency in haematological malignancies and none at all linked to myeloproliferative neoplasms (Khwaja, 2010, Kleppe and Levine, 2012). Despite this, there appears to be a clear link between increased JAK2 tyrosine kinase activity and aberrant activation of this pathway (James *et al.*, 2005). Constitutive activation of STAT5 in MPN as a result of deregulated JAK signalling

may result in a complex formed with the p85 subunit of PI3-kinase and also the scaffolding adapter protein Gab2 and results in increased Akt phosphorylation (Harir *et al.*, 2007).

1.6.2: Mitogen Activated Protein Kinase (MAPK) pathway

Mitogen activated protein kinase (MAPK) pathway, also referred to as the RAS/RAF/MEK/ERK pathway is another important cytokine signalling pathway through which JAK2 may impact. RAS lies upstream from this pathway and others, including the previously mentioned PI3K/Akt. On cytokine-receptor binding, RAS, a GTPase, can activate MAPK and trigger the signalling cascade. RAF migrates to the plasma membrane where it is activated and dimerises – active RAF, a serine/threonine kinase, can then phosphorylate MEK which in turn phosphorylates ERK (Chang *et al.*, 2003). ERK can act on both cytosolic and nuclear protein and impact transcriptional regulation, including a number of cell cycle regulatory proteins (Downward, 2003). There are several different mechanisms via which activated *JAK2*^{V617F} can affect the MAPK pathway. Phosphorylation of ERK 1 and 2 kinases has been identified in *JAK2*^{V617F} MPNs (James *et al.*, 2005, Levine *et al.*, 2005). There are three encoded forms of *RAS*, *H-, K-* and *N-RAS*. *K-RAS* plays a role in normal erythropoiesis, homozygous deletion in mice results in a fatal phenotype (Johnson *et al.*, 1997). Oncogenic gain of function mutations in *K-RAS* along with *N-RAS* are found in approximately 30% of myeloid malignancies (Kleppe and Levine, 2012). Although mutations in *RAS* are rarely observed in MPNs, they occur in 7-14% of post MPN acute myeloid leukaemia cases (Beer *et al.*, 2010, Kleppe and Levine, 2012), suggesting they may play an important role in leukaemic transformation. Deletions of *NF1*, a gene coding for a negative regulator of RAS signalling, have also been found in some patients with MF after previous PV or ET disease (Stegelmann *et al.*, 2010), indicating the potential impact this pathway has on MPN progression. When a constitutively active form of MEK was introduced to haematopoietic stem cells in mice it resulted in a myeloproliferative type disorder (Chung *et al.*, 2011).

1.7: Mutations in MPN

1.7.1: *JAK2*^{V617F} mutation

In 2005 several independent groups discovered a somatic gain of function mutation in *JAK2* (Baxter *et al.*, 2005, James *et al.*, 2005, Kralovics *et al.*, 2005, Levine *et al.*, 2005). A single valine to phenylalanine substitution at position 617 in the JH2 domain of *JAK2* results in constitutive kinase activity in the absence of cytokine binding to the membrane-bound receptor. This mutation has been found in nearly all (> 95%) cases of PV (Pardanani *et al.*, 2007) and up to 60% of ET and MF (Harrison and Vannucchi, 2016). Mutations in this domain, including *JAK2*^{V617F} in MPN patients, reduce Tyr570 phosphorylation and the ability to negatively control JH1 activity (Ungureanu *et al.*, 2011).

Since the discovery of the *JAK2*^{V617F} mutation, the key unanswered question has been how a single mutation can result in three distinct disease phenotypes. Animal model experiments using mice suggest the importance of the *JAK2*^{V617F} mutation in the disease phenotype (Li *et al.*, 2011). Lacout *et al.* (2006) demonstrated that over-expression of *JAK2*^{V617F} via retroviral transduction in mice resulted in a myeloproliferative disorder with a PV-like phenotype and secondary myelofibrosis. Transgenic and knock-in models also supported these findings and further research suggests a possible link between allele burden and the resulting MPN type disease. Shide *et al.* (2008) generated the first line of transgenic mice with *JAK2*^{V617F} ectopically expressed, which resulted in erythrocytosis, thrombocytosis and eventual progression to myelofibrosis. Another group reported similar findings expressing human *JAK2*^{V617F} in mice under the control of a tissue specific promoter (Xing *et al.*, 2008). Following on from these, Tiedt *et al.* (2008) generated a transgenic construct where it was possible to vary levels of *JAK2*^{V617F} expression. Higher levels of *JAK2*^{V617F} were found to correlate with a PV-like phenotype, whereas when *JAK2*^{V617F} levels were lower than the endogenous *JAK2*^{WT}, an ET-like phenotype was found (Tiedt *et al.*, 2008). A switch from ET to a PV-like phenotype was observed in mice with acquired *JAK2*^{V617F} homozygosity and the severity of the phenotype correlated with the

homozygous allele burden (Li *et al.*, 2014). These results along with the animal model studies support the gene-dosage hypothesis, where disease phenotype is determined by mutant allele burden proportion to wild-type (Passamonti and Rumi, 2009). PV patients have an overall higher percentage (48%) of mutant allele to wild-type versus ET (26%) and levels are even higher in secondary MF (74%) (Vannucchi *et al.*, 2008).

Although these studies may point to *JAK2*^{V617F} homozygosity being the principle driver of phenotype, it has also been shown that homozygous clones are present in large numbers of ET patients and undetected in a small number of PV patients (Godfrey *et al.*, 2012). This is noteworthy, since it was also shown that the principal distinguishing factor between the two phenotypes was the expansion of a dominant homozygous subclone in PV (Godfrey *et al.*, 2012). Disease phenotype does not appear to be simply determined by loss of heterogeneity but rather the clonal selection of a homozygous *JAK2*^{V617F} progenitor stem cell.

Gene expression profiles also differ between *JAK2*^{V617F} heterogenous erythroid cells from PV and ET patients, with increased STAT1 signalling observed in ET (Chen *et al.*, 2010) supporting a key role for STAT1 in determining disease phenotype in *JAK2*^{V617F} patients.

The role that the *JAK2*^{V617F} mutation plays in disease transformation is also not fully understood. *JAK2*^{V617F} only occurs in approximately 1% of cases of *de-novo* acute myeloid leukaemia (AML) with no known previous history of MPN, but is present in 50% of cases of AML secondary to MPN (Steensma *et al.*, 2006). It has also been shown that the majority of leukaemic blasts in transformed *JAK2*^{V617F} patients are negative for the *JAK2*^{V617F} mutation (Theocharides *et al.*, 2007). The significance of *JAK2* allele burden in blast transformation and overall survival has been widely studied. Several groups have shown no overall prognostic value in *JAK2*^{V617F} mutational status as a marker for either blast transformation or overall survival (Cervantes *et al.*, 2009, Tefferi *et al.*, 2005). However, others have claimed to show that low allele burden in MF is associated with poorer overall survival (Guglielmelli *et al.*, 2009, Tefferi *et al.*, 2008). More recently, with the discovery of the calreticulin (*CALR*) mutation

in ET and MF, poorer prognosis has been associated with *JAK2* mutations versus *CALR* (Tefferi *et al.*, 2014c). The role of mutated *JAK2* in disease progression is likely to be a consequence of molecular events which favour leukaemogenic events in the wild-type clones and, as mentioned earlier, the presence of additional mutations and the order in which they occur (Ortmann *et al.*, 2015).

1.7.2: Calreticulin (CALR)

Calreticulin is a protein located in the endoplasmic reticulum which sequesters Ca^{2+} (Luo and Lee, 2013). Knockout of calreticulin in mice results in death at the embryonic stage due to impaired cardiac development, which is dependent on Ca^{2+} signalling pathways (Guo *et al.*, 2002, Mesaeli *et al.*, 1999). It also plays a role in post-translational modifications of proteins in the ER, recognising N-linked glycans on glycoproteins and processing them for further folding or degradation (Michalak *et al.*, 2009, van Leeuwen and Kearse, 1996). In addition to its ER chaperone functions, calreticulin has also been found expressed at the surface of tumour and apoptotic cells where it is believed to promote phagocytosis (Gardai *et al.*, 2005, Obeid *et al.*, 2007).

In 2013, two independent groups discovered mutations in exon 9 of the *CALR* gene (Klampfl *et al.*, 2013, Nangalia *et al.*, 2013). Over 50 different mutations have been identified to date, with a 52-bp deletion (type 1 mutation) and a 5-bp insertion (type 2) most frequently seen (Cazzola and Kralovics, 2014). Mutations in calreticulin have been identified in 33% and 25% of ET and MF patients respectively (Andrikovics *et al.*, 2014). Evidence suggests that ET patients bearing a calreticulin mutation (as opposed to a *JAK2* or *MPL* mutation) have a lower risk of thrombosis (Rotunno *et al.*, 2014). Notably, *CALR* mutated ET patients do not have a polycythaemic transformation compared to 29% of *JAK2* mutated patients over a 15 year time period (Rumi *et al.*, 2014a). Myelofibrotic transformation risk over 15 years was reported to be higher (13.4%) in *CALR*-mutated ET versus *JAK2* (8.4%) and leukaemic transformation lower, 2.5% in *CALR* versus 4.3% in *JAK2*, although these differences were non-significant when adjusted for age (Rumi *et al.*, 2014b). Survival rates for *CALR*-mutated MF have been reported to be favourable compared to *JAK2* mutated or triple-negative

patients (Rumi *et al.*, 2014b, Tefferi *et al.*, 2014a), with median survival of 17.7 years for *CALR* versus 9.2 years for *JAK2*, 9.1 years for MPL and 3.2 years for triple-negative patients (Rumi *et al.*, 2014b).

Calreticulin mutants have been shown to activate the thrombopoietin receptor (MPL) and result in ligand-independent signalling through JAK-STAT, PI3K and MAPK pathways (Chachoua *et al.*, 2016). The mechanism has been demonstrated to be linked to activation by *CALR* mutants of N-glycosylation sites on the extracellular portion of the MPL receptor (Figure 6) (Chachoua *et al.*, 2016, Cazzola, 2016). Experiments in mice have also shown that the presence of MPL but not TPO is required for activation by *CALR* mutants (Marty *et al.*, 2015).

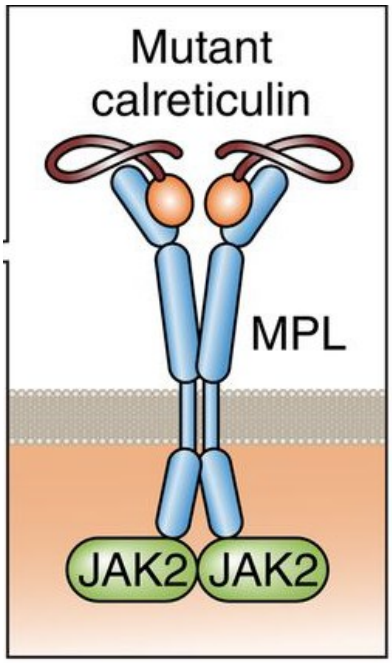


Figure 6: Diagram of pathogenic mechanism of CALR mutant MPN. Mutant calreticulin associates with MPL in the ER and is exported to the cell surface. This results in cytokine independent activation of MPL and JAK2 phosphorylation (Cazzola, 2016).

1.7.3: MPL

Megakaryocyte development is regulated by the binding of the cytokine thrombopoietin (TPO) to the membrane receptor MPL (Figure 7), and circulating levels of TPO are associated with platelet levels indicating a feedback loop mechanism between the two (de Graaf and Metcalf, 2011). In addition to its role in megakaryopoiesis, TPO signalling has also been implicated in haematopoietic stem cell (HSC) regulation, inhibition of TPO/MPL signalling results in reduction of the quiescent HSC population while stimulation with TPO increases the proportion of quiescent cells (Yoshihara *et al.*, 2007).

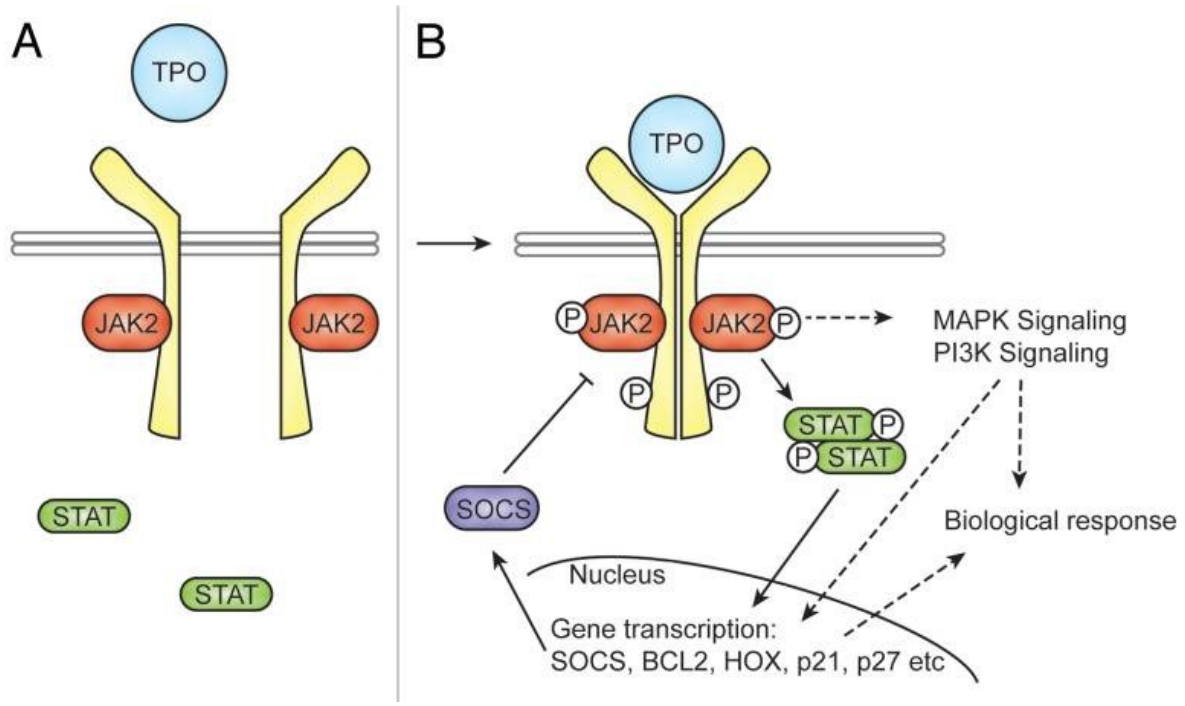


Figure 7: TPO/MPL signalling pathway. TPO binding causes dimerisation of the membrane-bound MPL receptor. This leads to phosphorylation and activation of JAK2 and STAT signalling molecules as well as other signalling pathways (MAPK/PI3K). Phosphorylated STAT translocates to the nucleus and results in altered gene transcription (de Graaf and Metcalf, 2011).

Mutations in exon 10 of the gene encoding the thrombopoietin receptor (MPL) have been recorded in between 3-10% of ET and MF cases (Tefferi, 2010, Vainchenker *et al.*, 2011). These mutations result

in the substitution of a tryptophan at position 515 to a leucine, lysine, asparagine, or alanine (Pikman *et al.*, 2006, Vainchenker *et al.*, 2011). Mutations in *MPL* can contribute to altered spatial confirmation of the receptor resulting in auto-phosphorylation of JAK2 and constitutive activation of the JAK/STAT pathway (Chaligné *et al.*, 2008). *MPL* mutations are not found in PV and are believed to enhance primary megakaryocyte proliferation (Tefferi, 2010).

1.7.4: Exon 12

Most of the 5% of *JAK2*^{V617F} negative PV cases have mutations located in exon 12 of *JAK2* (Passamonti *et al.*, 2011, Scott *et al.*, 2007). This is not directly located in the negative regulatory pseudokinase domain, but is located in a loop close to the interface between the pseudokinase and kinase domains (Scott, 2011). Patients with exon 12 mutations have higher haemoglobin levels but lower platelet and leukocyte counts at diagnosis than in *JAK2*^{V617F} patients, however overall survival, incidence of thrombosis and myelofibrotic/leukaemic transformations are similar (Passamonti *et al.*, 2011).

1.8: Transcription factor and epigenetic modifications

Downstream from cell surface signalling and the cytoplasmic pathways described previously, gene expression is regulated by transcription factors (TFs) within the nucleus binding to recognised sequences of DNA. Transcription factors also play an important role in determining the differentiation and expansion of haematopoietic stem cells into mature myeloid cells. The impact of TFs is further dependent on what lineage the cell is at and interactions with other transcription factors acting as repressors or enhancers of expression. TFs play a critical role in controlling differentiation of haematopoietic stem cells and determining lineage specification (Nakajima, 2011). A number of the most important transcription factors in haematopoiesis are discussed below.

1.8.1: *GATA1*

GATA1, plays an essential role in the development of normal erythroid cells and megakaryocytes (Crispino, 2005). It is expressed in primitive and mature erythroid cells, as well as megakaryocytes,

eosinophils, and mast cells (Ferreira *et al.*, 2005). Animal experimental studies have shown that *GATA*^{null} mice die during embryonic development, between embryonic day 10.5 and 11.5 due to severe anaemia (Fujiwara *et al.*, 1996). In adult mice, conditional knockout of *GATA1* results in impaired erythroid cell differentiation, where maturation is arrested at the proerythroblast stage (Gutiérrez *et al.*, 2008). Experiments where *GATA1* expression is knocked out in megakaryocyte progenitors have shown significantly reduced platelet levels (Shivdasani *et al.*, 1997). Mutations in *GATA1* are seen in almost all cases of acute megakaryoblastic leukaemia (AMKL) and transient myeloproliferative disorder (TMD) accompanying Down's syndrome (Greene *et al.*, 2003). Other studies have suggested that increased *GATA1* expression in AML is associated with a worse prognosis (Ayala *et al.*, 2009, Shimamoto *et al.*, 1995). GATA1 has three functional domains, two zinc fingers and an N-terminal activational domain (Crispino, 2005). One of the zinc fingers (C) is responsible for binding to the consensus sequence on the DNA. The other zinc finger (N) also has DNA binding activity, specific to a palindromic consensus sequence, as well as being important for the recruitment and binding of a co-factor, friend of GATA1, (FOG1) (Tsang *et al.*, 1997).

1.8.2: FOG1

FOG1 has been identified as being expressed in erythroid and megakaryocytic progenitors and is essential for normal erythropoiesis and megakaryopoiesis (Tsang *et al.*, 1998). In the mast cell lineage, *GATA1* is expressed independently to *FOG1* (Cantor *et al.*, 2008). Experiments with *FOG1* constitutively expressed in progenitor cells led to a block on eosinophil production, and forced expression in eosinophils resulted in a loss of specific eosinophilic cell surface markers and a reverting to a multipotent progenitor phenotype (Querfurth *et al.*, 2000). FOG1 has also been shown not only to have a synergistic effect on gene expression with GATA1 but is also responsible for inhibiting expression of some genes, most likely through the recruitment of co-repressors such as CtB2 (Katz *et al.*, 2002). *GATA1* has been found to be overexpressed in ET and PV but not in other MPNs, and this is independent of the *JAK2*^{V617F} mutation (Rinaldi *et al.*, 2008). CD61+ cells from myelofibrosis patients

had similar *GATA1* transcript levels to controls but significantly reduced protein expression (Vannucchi *et al.*, 2005).

1.8.3: *GATA2*

GATA2 also plays a critical role in haematopoiesis, in particular during the early stages of HSC proliferation and survival but is dispensable during later erythroid and myeloid terminal differentiation (Tsai and Orkin, 1997). It binds an overlapping set of genes with its related family member, GATA1, at several distinct sites. The mechanism of switching from GATA2 to GATA1 occupation at these locations may act as a balance between proliferation and differentiation (Doré *et al.*, 2012). Mutations in *GATA2* have been linked to chronic myeloid leukaemia and transformation to AML (Zhang *et al.*, 2008) and overexpression has been associated with poorer prognostic outcomes in AML (Vicente *et al.*, 2012). Inherited missense mutations in the DNA-binding region of GATA2 have been linked to a predisposition for the development of familial MDS/AML (Hahn *et al.*, 2011).

1.8.4: *PU.1*

PU.1 is an ETS transcription factor family member and is encoded by the *Sfp1* (*Sp-1*) gene (Burda *et al.*, 2010). Along with GATA1, it is responsible for lineage stage determination in early HSC (Figure 8). PU.1 transgenic mice were found to develop a multistep erythroleukaemia, where differentiation was partially blocked at the pro-erythroblast level (Moreau-Gachelin *et al.*, 1996). Levels of PU.1 were found to be raised in MPN patients and this correlated with the $JAK2^{V617F}$ allele burden. In cellular models, overexpression of $JAK2^{V617F}$ in HEL cells resulted in increased Sp1 expression but this was not observed in wild type *JAK2* expression in K562 cells. In addition, it was also demonstrated that inhibition of ABL1 with imatinib reduced PU.1 levels in K562 but not *BCR-ABL1* negative / $JAK2^{V617F}$ positive HEL cells, suggesting that this transcription factor may be a common downstream target for both JAK2 and ABL1 oncogenic signalling (Irino *et al.*, 2011).

1.8.5: *NFE2*

Nuclear factor erythroid-2 (NFE2) is a haematopoietic transcription factor and is essential for normal platelet formation (Shivdasani *et al.*, 1995). Overexpression of *NFE2* has been found in the vast

majority of PV patients (Goerttler *et al.*, 2005). Increased *NFE2* is also responsible for delayed erythroid maturation and results in an increase in the number of mature erythroid cells deriving from a single progenitor (Mutschler *et al.*, 2009). It has also been found over expressed in other MPN patients, independent of *JAK2*^{V617F} mutation status (Goerttler *et al.*, 2005, Wang *et al.*, 2010). Analysis of the promoter sequence upstream from *NFE-2* has suggested a potential role for AML1 in modulating the levels of this transcription factor. AML1 binds the *NFE2* promoter at three locations and increased *NFE-2* and *AML1* levels are found in MPN patients (Wang *et al.*, 2010). Furthermore, point mutations in *AML1* have been implicated in leukaemic transformation in MPN patients (Ding *et al.*, 2009), suggesting that it, along with *NFE2,* may play a role in the pathogenesis of MPNs.

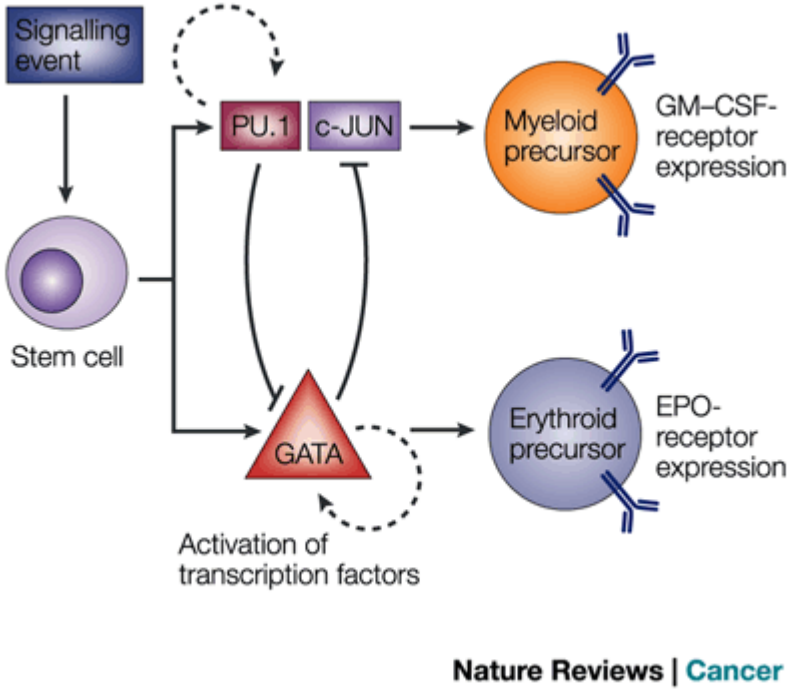


Figure 8: Diagram showing role of GATA-1 and PU.1 transcription factors have on lineage determination (Tenen, 2003).

There are three basic epigenetic mechanisms which interact with each other to affect gene expression in the nucleus:

1) DNA methylation.

2) Histone modifications including acetylation, methylation, phosphorylation and ubiquitination.

3) Chromatin remodelling.

1.8.6: DNA methylation

Studies on individual genes (*SOCS1, SOCS3,* and *PTPN6)* involved in negatively regulating *JAK2* have shown an increase in methylation of *SOCS1* in ET patients compared to control (Födermayr *et al.*, 2012). However, no differences were seen for PV patients and methylation of either *SOCS* or *PTPN* did not correlate with any clinical outcome (Födermayr *et al.*, 2012). Another group utilised a genome wide array and found an aberrant methylation pattern for the MPNs centred around a gene network for NF-κB (Pérez *et al.*, 2013). Interestingly, it was also noted that this difference was increased for transformed MPNs, suggesting that DNA methylation may play a role in the pathogenesis of the disease.

Mutations in the gene for DNA methyltransferase-3 (*DNMT3A*) have also been identified in myeloproliferative neoplasms (Stegelmann *et al.*, 2011). *DNMT3A* has previously been found at a high frequency (22%) and is associated with a poor prognosis in acute myeloid leukaemia patients (Ley *et al.*, 2010). The role of *DNMT3A* may be in the advanced phase of the myeloproliferative disease.

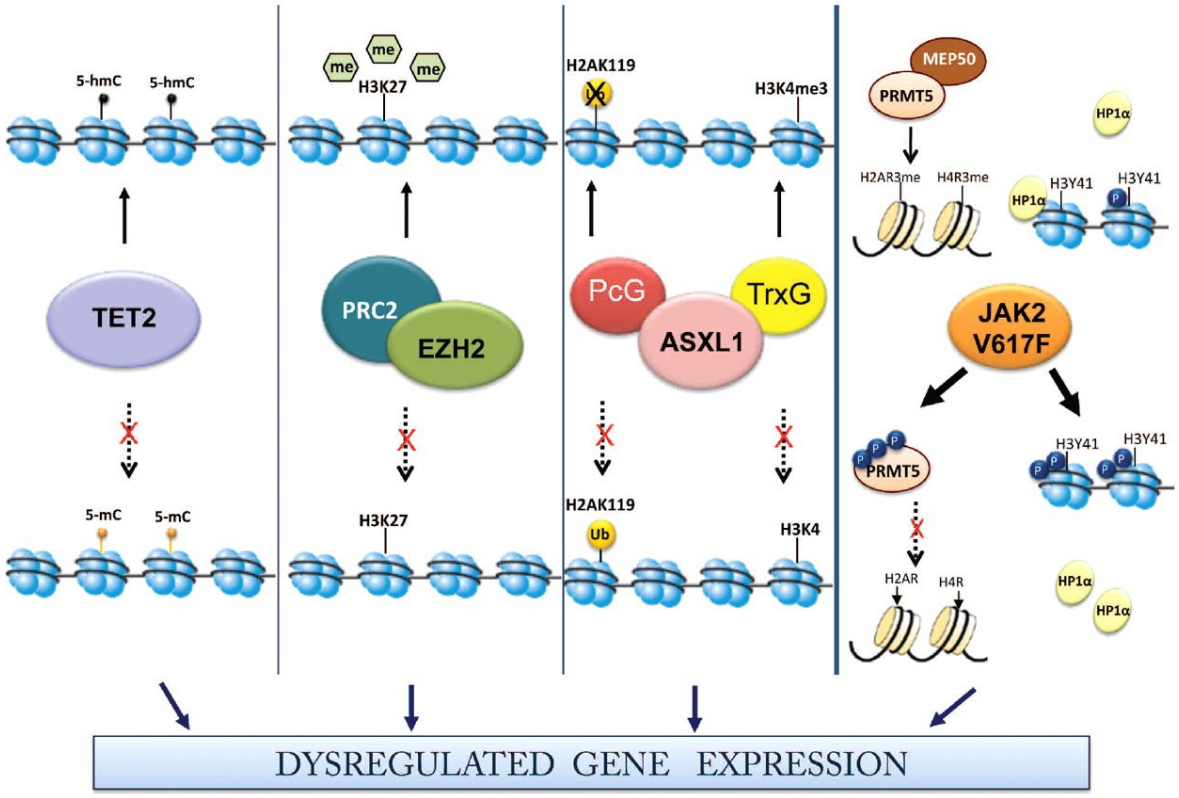


Figure 9: Various mutations observed in MPNs and their effect on epigenetic gene regulation (Vannucchi and Biamonte, 2011).

1.8.7: Histone modifications and chromatin remodelling

JAK2 has a role in modulating signalling cascades in the cytoplasm, while it has also been shown to be responsible for chromatin modification. JAK2 can directly phosphorylate Tyr41 on histone H3 (H3Y41) (Figure 10) and in doing so prevents the binding of the heterochromatin protein 1 alpha (HP1α) (Dawson *et al.*, 2009). $JAK2^{V617F}$ has been shown to translocate to the nucleus of CD34+ cells of MPN patients but not to granulocytic, megakaryocytic or erythroid cells (Rinaldi *et al.*, 2010). This could also be seen when K562 cells were induced via transfection to express $JAK2^{V617F}$ and the reverse (relocation to the cytoplasm) observed when cells were treated with a specific JAK2 inhibitor (Rinaldi *et al.*, 2010).

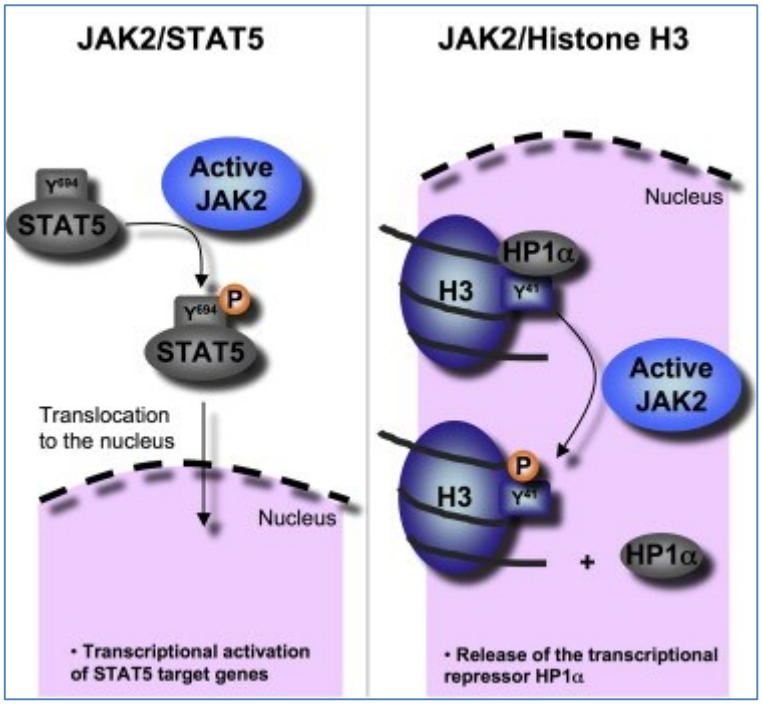


Figure 10: Activation of JAK2 and translocation to the nucleus. Active JAK2 can effect gene expression via phosphorylation of the histone protein, H3Y41. This results in the release of the repressor, HP1α (Sattler and Griffin, 2009).

Protein arginine methylation is a process by which protein-nucleic acid interactions can be modified. Liu *et al.,* (2011) demonstrated that mutations in JAK2, including *JAK2*^{V617F} and *JAK2*^{K529L} (occurring in exon 12) resulted in the phosphorylation of protein arginine N-methyltransferase 5 (PRMT5) (Liu *et al.*, 2011). PMRT5 is a type 2 arginine methyltransferase, and along with a WD40 repeat containing MEP50 protein and plCln, forms a 20S complex known as the methylosome (Friesen *et al.*, 2001). This regulates methylation of arginine residues at several histones, including H2A, H3 and H4 (Antonysamy *et al.*, 2012). The binding of the mutated JAK2 to PRMT5 was found to be stronger than in normal JAK2 and reduced the ability of PRMT5 to form a complex with MEP50 (Figure 9). PRMT5 was also shown to down-regulate haematopoietic stem cell expansion and erythroid differentiation (Liu *et al.*, 2011).

Epigenetic modifications are also a feature of *JAK2*^{V617F} negative MPN cases. Gene expression can be altered via chromatin remodelling or DNA methylation and several mutations have been identified

that have the ability to alter these processes. These can occur with or without the presence of the previously described *JAK2* tyrosine kinase mutations (Vannucchi and Biamonte, 2011). TET2 affects DNA methylation through the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (Figure 9) (Ko *et al.*, 2010). Mutations in *TET2* are observed in a wide range of haematological cancers (Delhommeau *et al.*, 2009).

ASXL1 is required for normal haematopoiesis and mutations in exon 12 of this gene are observed in a number of myeloid malignancies (Carbuccia *et al.*, 2009, Schnittger *et al.*, 2013). ASXL1 forms a complex with other proteins and affects gene expression (positive and negative) by altering chromatin configuration (Figure 9) (Vannucchi and Biamonte, 2011). Mutations have been identified at a frequency of 13% in PMF, 23% in post-PV/ET MF and 18% in blast-phase MPN (Tefferi *et al.*, 2011).

IDH1 and 2 affect gene expression by regulating histone and DNA methylation levels (Sasaki *et al.*, 2012), and have been identified in a high incidence of patients suffering from blast phase MPN but also in a low frequency in chronic phase MPN (Pardanani *et al.*, 2010), although the exact mechanisms, if they exist, for leukaemic transformation are not yet fully understood.

1.9: Drug development & targets

1.9.1: Allogeneic stem cell transplantation

Allogeneic haematopoietic stem cell transplantation (ASCT) is currently the only treatment that is proven to be fully curative (Gupta *et al.*, 2014), however the age profile for many MPN sufferers is such that the risks outweigh any potential benefits over the treatments listed below.

1.9.2: Hydroxyurea

Hydroxyurea (hydroxycarbamide) is the current first-line therapy of choice for treatment of MPN patients at high risk of thrombotic events (Harrison *et al.*, 2005), the leading cause of morbidity and mortality in essential thrombocythaemia and polycythaemia vera (Falanga and Marchetti, 2012). The anti-myeloproliferative effects of hydroxyurea occur through inhibition of the ribonucleotide reductase enzyme, which is responsible for the catalytic production of deoxyribonucleotides from ribonucleotides (Hong and Erusalimsky, 2002). One potential mechanism of action has been proposed through the production of nitric oxide free radicals which target a tyrosyl free radical found in the reductase enzyme (Lepoivre *et al.*, 1994). Inhibition of cell proliferation occurs primarily during the S phase or late G1 phase (Bertoli *et al.*, 2013).

1.9.3: Anagrelide

The reduction of intracellular deoxynucleoside triphosphate pools is a double-edged sword, since DNA repair mechanisms are also possibly affected (Aye *et al.*, 2015). This mutagenic and carcinogenic potential has been observed in animal and cellular models (Santos *et al.*, 2011), although no significant link to date has been proven in human patients given therapeutic doses. Nevertheless, the theoretical risk has led to anagrelide being proposed as an alternative therapy in ET patients (Emadi and Spivak, 2009), and also in use where patients are non-responsive to hydroxyurea treatment (Finazzi, 2012). Unlike hydroxyurea, whose action is anti-proliferative, anagrelide acts through the inhibition of megakaryocytopoeisis (Solberg *et al.*, 1997). The mechanism of inhibition is not fully understood, although anagrelide does act as a potent phosphodiesterase type III (PDE3) inhibitor (Gresele *et al.*, 2011). Phosphodiesterases are enzymes that are responsible for catalysing the hydrolysis of

intracellular second messengers, cyclic adenosine 3, 5-monophosphate (cAMP) and cyclic guanosine 3, 5-monophosphate (cGMP), to the inactive 5'-AMP and 5'-GMP forms (Gresele *et al.*, 2011). PDE3 is expressed in platelets, as well as vascular smooth muscle and heart cells, and acts mainly on cAMP (Gresele *et al.*, 2011, Omori and Kotera, 2007). Increased concentration of cAMP can activate protein kinase A (PKA) by phosphorylation, which in turn can activate a signalling cascade resulting in the regulation of a number of genes (Zambon *et al.*, 2005).

1.9.4: Acetylsalicylic acid

Acetylsalicylic acid (aspirin) is used alongside the conventional therapies listed previously as a preventative measure against thrombotic complications associated with myeloproliferative neoplasms. The major risk with this treatment is an increased risk of bleeding. The mechanism of action is through the suppression of platelet thromboxane A2 (TXA2) synthesis via inactivation of cyclo-oxygenase-1 (COX-1) (Tefferi, 2012).

1.9.5: Interferon-α

Experiments using *JAK2*^{V617F} mice have demonstrated that prolonged treatment with interferon α results in the reduction of a number of symptoms, including normalisation of haemoglobin levels, reduction of white cell count and extramedullary haematopoiesis (Lane and Mullaly, 2013). It was also noted that there was a reduction in the *JAK2*^{V617F} allele burden over time (Lane and Mullaly, 2013). Clinical studies in humans have also shown similar haematological and molecular responses, including the reduction of the *JAK2*^{V617F} clone (Quintás-Cardama *et al.*, 2009). The mode of action of IFN-α is via surface bound receptors activating JAK1 or TYK2 at the cell surface; these in turn can phosphorylate and dimerise STAT proteins in the cytoplasm resulting in translocation to the nucleus and transcription of a number of pro-apoptotic and cell growth inhibition genes (Trinchieri, 2010). The experiments described above suggest that IFN-α is acting preferentially on the mutant clone. It has also been shown that *JAK2*^{V617F} cells treated with IFN-α have a number of cell cycle genes up-regulated both before and after treatment (Lane and Mullaly, 2013). This may provide an explanation as to the preferential responsiveness to IFN-α shown by *JAK2*^{V617F} cells. However IFN-α is, for most patients,

poorly tolerated over a prolonged period (Quintás-Cardama *et al.*, 2009, Lane and Mullaly, 2013), and cannot be considered as a curative measure on its own.

1.9.6: Ruxolitinib and JAK2 inhibitors

The discovery of the BCR-ABL fusion protein and identification of its tyrosine kinase activity paved the way for development of specific drugs targeting the src homology (SH1) domain on ABL. This has been identified as a key target in promoting leukaemogenicity and oncogenic transformation. One such drug, imatininb, works by competing for binding at the SH1 domain. With the identification of the central role of JAK2 signalling and constitutive activation in *BCR-ABL1* negative MPN through mutant *JAK2* and other mutations, it has been hoped that similar TK inhibiting drugs could be developed, with a view to selectively targeting JAK2 activity. The first, and to date only, approved drug for the treatment of MPN to make it to market has been ruxolitinib (INCB018424) (Mesa *et al.*, 2012), which in addition to targeting JAK2 also inhibits JAK1 activity and also has a smaller effect on TYK2 and JAK3 (Quintás-Cardama *et al.*, 2010). The results from published clinical trial data suggests that ruxolitinib is effective in reducing splenomegaly and overall quality of life is improved (Harrison *et al.*, 2016, Vannucchi *et al.*, 2015a). It has also been shown to improve overall survival in MF patients versus either best available therapy or placebo irrespective of *JAK2* mutational status (Harrison *et al.*, 2016, Vannucchi *et al.*, 2015a).

It would seem obvious that direct targeting of $JAK2^{V617F}$ might be more effective than pan-JAK inhibitors. However the $JAK2^{V617F}$ mutation occurs outside the ATP binding domain on the JAK2 enzyme meaning ATP-competitive inhibitors cannot distinguish between normal and mutant forms (Verstovsek, 2009). Along with the previously mentioned drugs, several others are in development and undergoing various stages of clinical trials. These are primarily JAK-type inhibitors similar to ruxolitinib, although notably one, TG101348 (SAR302503), has recently been withdrawn due to safety concerns (Pardanani *et al.*, 2015).

1.9.7: Givinostat and histone deacetylase inhibitors

The use of histone deacetylase (HDAC) inhibitors are another potential avenue for therapy design. Acetylation and de-acetylation of histone proteins provides a mechanism for controlling chromatin remodelling and the expression of a number of genes (Ropero and Esteller, 2007). To date, eighteen different mammalian HDAC enzymes have been discovered and are classed according to similarities in structure. Class 1 HDACs, the most well studied group, include HDAC 1, 2, 3 and 8, and are ubiquitously expressed (Haberland *et al.*, 2009). Class 2 can be subdivided into two subgroups, 2a (HDAC 4, 7 and 9) and 2b (HDAC 6 and 10) (Haberland *et al.*, 2009). Class 4 include the sirtuins, SIRT 1-7 (Delcuve *et al.*, 2012, Ropero and Esteller, 2007). Sodium butyrate, a HDAC inhibitor, was shown to increase the levels of SOCS 1 and 3 in K562 and HEL cells and subsequently reduce the levels of JAK2, STAT3, STAT5 and their phosphorylated forms (Gao *et al.*, 2013). Givinostat (ITF2357), a novel HDAC inhibitor is currently in phase 2 clinical trials for PV patients unresponsive to hydroxyurea (Finazzi *et al.*, 2013), and in a previous clinical trial reduced symptoms such as splenomegaly and pruritus in the majority of PV/ET and some MF patients (Rambaldi *et al.*, 2010). Givinostat has been shown to down-regulate expression of a number of genes for transcription factors involved in differentiation along the erythroid, megakaryocytic and myeloid lineages (Amaru Calzada *et al.*, 2012). These include *TAL1, NFE2* and *c-MYC*, of which both the latter two had protein and mRNA levels downmodulated in response to givinostat treatment. The effect on *NFE2* was shown to be directly via acetylation of histone H3 protein on distal and proximal promoter sites (Amaru Calzada *et al.*, 2012).

1.9.8: Heat shock protein inhibitors

Targeting heat shock proteins may provide another pathway in treating MPN. Heat shock proteins are chaperone proteins in the cell and provide a protective effect from degradation. JAK2 has been shown to associate with one heat shock protein, HSP90, and treatment with an inhibitor, PU-H71 reduces proliferation and results in faster proteasomal degradation (Marubayashi *et al.*, 2010). In addition, treatment with PU-H71 reduced MPL^{W515L} mutant burden in murine models (Marubayashi *et al.*, 2010). Another group found that heat shock protein inhibition had a synergistic effect when used as a co-

treatment alongside JAK2 tyrosine kinase inhibitors. Fiskus *et al.* (2011) demonstrated that AUY922, a HSP90 inhibitor, when used with TG101209 induced more apoptosis and inhibited JAK2 signalling than either drug alone. This treatment was shown to be selectively more active against primary MF-MPN haematopoietic stem cells than normal non-mutated ones (Fiskus *et al.*, 2011). A further benefit observed was that the treatment with the HSP90 inhibitor could overcome previous resistance to tyrosine kinase inhibition treatment. Panobinostat, a class 1 and 2 HDAC inhibitor, was able to reduce *JAK2* mRNA expression and prevent JAK2 binding with HSP90 in a MPN cell line (Wang *et al.*, 2009). As before, co-treatment with a tyrosine kinase inhibitor resulted in an enhanced effect (Wang *et al.*, 2009).

1.10: Clinical Trials

Currently there are a number of ongoing or recently completed clinical trials investigating the safety and efficacy of novel therapies, as well as assessment of current treatment modalities in MPNs. A brief summary of some of the major trials are detailed below.

1.10.1: PT-1

The PT-1 trial was a large multi-centre study looking at patients with essential thrombocythaemia (ClinicalTrials.gov #NCT00175838). The major aims for this study were 1) Examine the incidence of thrombosis and major haemorrhage in low-risk patients receiving aspirin only, 2) Assess whether hydroxyurea with aspirin reduces risk of thrombosis and haemorrhage in intermediate risk patients, 3) Impact of treatment selection on quality of life and 4) Follow-up of high-risk patients (> 60 years old) to assess frequency of thrombosis and haemorrhage events, including patients receiving anagrelide. Secondary objectives for the study included assessment whether treatment choice affected the rate of leukaemic or myelofibrotic transformation.

Superiority of hydroxyurea (with aspirin) over anagrelide in controlling vascular events was demonstrated in high-risk patients with ET (Harrison *et al.*, 2005). This contradicts the results of another study, ANAHYDRET (ClinicalTrials.gov #NCT01065038), which claimed non-inferiority of anagrelide compared to hydroxyurea treatment (Gisslinger *et al.*, 2013). The major differences between the two studies involved aspirin as a co-treatment in the PT-1 patients and the diagnostic criteria used. Patients in the PT-1 group were selected using the Polycythaemia Vera Study Group (PVSG) as opposed to the WHO criteria in the ANAHYDRET study.

1.10.2: COMFORT I/II

Ruxolitinib, a JAK2 inhibitor, has been examined in patients with myelofibrosis in the COMFORT I study (ClinicalTrials.gov #NCT00952289). Reductions in constitutive symptoms, including splenomegaly and improvements in quality of life were demonstrated over placebo (Verstovsek *et al.*, 2012b). The COMFORT II study further showed these improvements over best available therapy (BAT) in addition to increased overall survival rates in patients receiving ruxolitinib (Cervantes *et al.*, 2013) (ClinicalTrials.gov #NCT00934544).

A number of studies have been completed or are ongoing to evaluate the utility in treating PV or ET with ruxolitinib in patients who are resistant or intolerant to hydroxyurea therapy. These include the MAJIC trial (UKCRN ID: 11941) and RESPONSE (ClinicalTrials.gov # NCT01243944). The RESPONSE study showed ruxolitinib was superior to standard therapy in reducing symptoms associated with PV, including splenomegaly, as well as controlling the haematocrit (Vannucchi *et al.*, 2015b). The RELIEF trial (ClinicalTrials.gov #NCT01632904) compared ruxolitinib directly against hydroxyurea treatment in PV patients.

In a phase II clinical trial (INCB18424-256, ClinicalTrials.gov #NCT00726232) examining safety and efficacy of ruxolitinib in PV and ET, three patients were shown to have complete molecular remission in $JAK2^{V617F}$ allele burden after 5 years (Pieri *et al.*, 2015).

1.10.3: PERSIST-I

Pacritinib (SB1518) is a novel pyrimidine based small molecule with inhibitory activity against *JAK2*^{WT} (IC_{50} = 22 nM), and *JAK2*^{V617F} (IC_{50} = 19nM) (Hart *et al.*, 2011a, William *et al.*, 2011). It also has equipotent activity (IC_{50} = 23nM) against fms-like tyrosine kinase-3 (*FLT3*), a gene commonly found to be mutated in AML patients(Hart *et al.*, 2011b). PERSIST-I is an ongoing study examining pacritinib versus BAT in myelofibrosis patients (ClinicalTrials.gov #NCT00745550). Recent results suggest that pacritinib is effective in reducing spleen volume (31% of patients achieved a ≥ 35% decrease by MRI / 42% had decrease ≥ 50% by physical examination), MF symptoms (with the exception of fatigue) were also reduced (Komrokji *et al.*, 2015).

1.10.4: CYT3817 / Momelotinib

CYT3817 is an aminopyrimidine derivative, ATP-competitive inhibitor of JAK1, JAK2 and TYK2 (Tyner *et al.*, 2010, Pardanani *et al.*, 2009). It inhibits growth of cell lines (HEL and Ba/F3) containing either the *JAK2*^{V617F} (IC_{50} = 1500 nM) or *MPL*^{W515L} (IC_{50} = 200 nM) mutations but has minimal effect on the BCR-ABL cell line K562 (IC_{50} = 58000 nM). Phase I/II studies with CYT3817 in MF patients demonstrated that 46% had a spleen reduction ≥ 50% and 68% of previously transfusion dependent patients became transfusion independent (Gotlib *et al.*, 2013). This effect on anaemia is notable in contrast with that seen in other JAK inhibitors, including ruxolitinib (Harrison *et al.*, 2012, Verstovsek *et al.*, 2012b). Currently CYT3817 is undergoing a phase III clinical trial (ClinicalTrials.gov #NCT02101268), the primary endpoint for the trial is splenic response rate (> 35%) at week 24.

1.10.5: PEGASYS

Interferon alpha (IFN-α) is known to reduce the colony-forming ability of erythrocyte, granulocyte and megakaryocyte progenitors in MPN patients (Kiladjian *et al.*, 2008). A pegylated form of IFN-α (Peg-IFN-α-2a), which has lower toxicity and a better pharmacokinetic profile, has been shown to have high levels of molecular and haematological responses in previously untreated ET and PV patients (Quintás-

Cardama *et al.*, 2009). This included a subset of patients (6% and 14% for ET and PV respectively) where *JAK2*^{V617F} was completely undetectable after treatment. A phase III trial of pegylated interferon (+ aspirin) versus hydroxyurea (+ aspirin) is currently ongoing (ClinicalTrials.gov #NCT01259856).

1.11: Summary

Although key advances have been made in the last decade of our understanding of the molecular genetics of the classical myeloproliferative neoplasms, it is debatable whether this has been translated into a significant increase in patient quality of life and reduced mortality, especially for myelofibrosis, and morbidity. None of the existing treatments provide a fully curative option, and only one JAK2 tyrosine kinase inhibitor has been released to date.

Combination therapy may prove, in the short term at least, the most effective development in the management of patients with MPN. Treatment with both conventional therapies, such as hydroxyurea, and HDAC inhibitors may have synergistic apoptotic effects as seen in cell model studies (Amaru Calzada *et al.*, 2013), and in clinical trials (Finazzi *et al.*, 2013). As well as targeting the different stages of the classical JAK/STAT pathway, other signalling cascades affected by deregulated JAK2, especially those described earlier (PI3K/Akt and MAPK), could provide attractive targets for drug development or already have specific inhibitors that could be utilised. However, it is also important to note that in *CALR* cell models, synergy between JAK2 and PI3K inhibitors was not observed compared to JAK2 cell models (Chachoua *et al.*, 2016). Phenotype differences between the three main Philadelphia negative MPNs may be in part explained by changes at the nuclear level modulated by differential transcription factor and micro RNA expression profiles. These epigenetic changes may provide a key target for treatment or in monitoring disease progression.

The impact of the increased number of treatments, each targeting different pathways at different cell cycle stages means that a key understanding of the molecular events in MPNs are more important than ever. This will apply in the development of new drugs as well as tailoring current regimes to fit

the disease profile. In the absence of a treatment to fully remove the disease allele, understanding the mechanisms via which MPNs progress and identifying potential key steps in the transformation of the disease will be crucial in improving patient quality of life.

1.12: Aims

This project aims to investigate the molecular mechanisms of deregulated JAK2 signalling in MPNs and identify changes to key pathways involved in haematopoiesis. It is hoped that these studies will identify potential targets for MPN treatment and improve our understanding into how different disease phenotypes can arise from mutations affecting the JAK/STAT signalling pathway. Three approaches will be used in this project:

1) Investigate *GATA1* expression in MPN patients.
2) Examine the role of *GATA1* in cell-line models.
3) Proteomics to study effect of JAK2 signalling in cell models and MPN patients.

CHAPTER 2: Methodology

2.1: Isolation of Peripheral Blood Mononuclear cells (PBMCs) from human donors

Two patient cohort groups were recruited for this research project. Ethical approvals for the study are documented in the appendices.

Cohort 1: GATA1 Expression levels in peripheral blood of patients with ET [NHS Ethics: 12/EM/0350]

Patients diagnosed with essential thrombocythaemia according to the 2008 WHO criteria were recruited for this study.

Exclusion criteria for this study were:

- Patients with any other myeloproliferative disorder.

Written informed consent was obtained from all patients before peripheral blood samples were taken. Sample collection (peripheral whole blood) was carried out by a trained phlebotomist using venepuncture.

Peripheral blood was obtained from ET patients (n = 36) and healthy control donors (n = 7). Phosphate buffered saline solution was prepared by dissolving one Oxoid™ PBS tablet (Oxoid, Basingstoke, UK) in 100 mL water and adding 200 µL of a 0.5 M EDTA (Fisher Scientific, Loughborough, UK) pH 8.0 solution. The PBS/EDTA solution was adjusted to pH 7.4. Whole blood was diluted with the PBS/EDTA solution (1 in 4 dilution) and layered over Ficoll-Paque Premium 1.077 g/m (GE Healthcare Life Sciences, Buckinghamshire, UK) in a 50 mL Falcon tube. The tube was centrifuged for 40 minutes in a swing bucket rotor centrifuge (Model: Allegra X-15R, Manufacturer: Beckman-Coulter, High Wycombe, UK) at 450 x g with the brake switched off and acceleration set to minimum. Temperature was set at 20ºC. After centrifugation, the PBMC layer at the interface between the Ficoll-Paque and plasma layers was carefully extracted using a Pasteur pipette and transferred to a clean 15 mL Falcon tube. 10 mL of PBS/EDTA was added to the PBMCs and the tube was centrifuged for 10 minutes at 300 x g with brake

switched on. Supernatant was discarded after centrifugation and cells were resuspended with 10 mL of PBS/EDTA. This was then centrifuged for 10 minutes at 200 x g (brake on). Supernatant was discarded and cells were resuspended in appropriate volume (5 mL) of PBS and a cell count was taken using 0.4% trypan blue (Sigma-Aldrich, Poole, UK).

Cohort 2: JAK2 in Myeloproliferative Neoplasms [NHS Ethics: 14/NW/1444]

Patients with a myeloproliferative neoplasm, either PV, ET, or MF according to the WHO 2008 diagnostic criteria were recruited for this study. All patients were 18 or above and had been recently diagnosed with an MPN or on cytoreductive therapy (hydroxyurea or anagrelide) at the time of sampling.

Exclusion criteria for this study were:

- Patients on ruxolitinib.
- Patients who had received chemotherapy, any investigational drug or had undergone major surgery within the last four weeks.
- Female patients who were pregnant or breast-feeding.
- Patients with any other myeloproliferative disorder.

Written informed consent was obtained from all patients before peripheral blood samples were taken.

Peripheral blood (4 mL) was diluted with equal volume of Iscove's MDM + 2% BSA (Stemcell Technologies, Vancouver, Canada) and inverted gently to mix. This was layered over 5 mL of Ficoll-Paque Premium 1.077 g/m in a 15 mL Falcon tube. The tube was centrifuged for 30 mins at 400 x g with brake off and acceleration at minimum setting in a swing bucket rotor centrifuge (Model: Allegra X-15R, Manufacturer: Beckman-Coulter, High Wycombe, UK). A clean Pasteur pipette was used to transfer cells from the interface between the plasma and Ficoll layers to a fresh 15 mL tube. 10 mL of IMDM + 2% FBS was added to the tube containing the isolated cells. The tube was centrifuged for 10 mins at 300 x g with the brake on. Supernatant was carefully discarded and pellet resuspended in 10

mL of IMDM + 2% FBS. Tube was centrifuged for 10 mins at 300 x g, the supernatant removed and resuspended in 2 mL of IMDM + 2% FBS. A cell count was performed using trypan blue assay.

An aliquot (300 µL) of a 10 X cell solution (2 x 10^6 cells/mL) was added to 3 mL of MethoCult methylcellulose media, H4034 Optimum (Stemcell Technologies, Vancouver, Canada). Ruxolitinib (100 and 250 nM) was added to the methylcellulose media. Vehicle control, dimethyl sulfoxide (DMSO), was also added to the methylcellulose media, at the same concentration as that of the highest drug concentration (250 nM) was dissolved in. The mixture was gently rolled and vortexed briefly to mix before allowing to settle. 1.1 mL of media was applied to each colony dish using a blunt end needle and syringe (Stemcell Technologies, Vancouver, Canada). Cells were incubated at 37ºC in a 5% CO_2 incubator for 14 days before colony types were identified and counted. BFU-E colonies were isolated and collected in 5 mL ice-cold PBS and centrifuged (300 x g for 3 minutes). Supernatant was discarded and pellet was resuspended in 1 mL ice-cold PBS. A cell count in 0.4% trypan blue was taken. The sample was then centrifuged at full speed in a microcentrifuge. Supernatant was aspirated completely and the pellet stored at -80ºC.

2.2: Extraction of RNA from PBMCs using TRIzol® method

PBMC cells (1 x 10^6) in PBS were spun down and supernatant was discarded to leave pellet in a 1.5 mL eppendorf. The PBMC pellet was solubilised in 1 mL TRIzol® reagent (Thermo-Fisher, Paisley, UK). 200 µL of chloroform (Sigma-Aldrich, Dorset, UK) was added to the cells in TRIzol® and the mixture was shaken vigorously by hand for 15 seconds. The eppendorf was left to settle for 3 minutes before centrifugation (Model: Micro Star 17R, Manufacturer: VWR, Lutterworth, UK) at 12,000 x g for 15 minutes at 4ºC. After centrifugation, the upper aqueous layer was removed and placed in a clean RNA/DNA free eppendorf. 0.5 µL of glycogen (20 µg/µL) (Life Technologies, Paisley, UK) was added to the aqueous phase followed by 500 µL of analytical grade isopropanol (Fisher Scientific, Loughborough, UK). The mixture was incubated at room temperature for 10 minutes. After incubation, the tube was centrifuged for 10 minutes at 12,000 x g at 4ºC. Supernatant was removed and pellet was washed with

75% ethanol (Fisher Scientific, Loughborough, UK) in DEPC water (Sigma-Aldrich, Poole, UK). Pellet was centrifuged at 7500 x g for 5 minutes at 4ºC. Supernatant was removed and pellet was allowed to air-dry for 10 minutes before dissolving in 50 µL of nuclease-free water (Sigma-Aldrich, Poole, UK). The tube was incubated at 55-60ºC on a block heater (Model: SBH130D/3, Manufacturer: Stuart, Stone, UK) for 10 minutes.

2.3: Cleanup of RNA obtained using the TRIzol® extraction method

Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) was used to clean-up RNA after phenol-chloroform extraction. The sample was adjusted to 100 µL total volume with RNAse free water. 350 µL of buffer RLT from the RNeasy kit was added to the 100 µL sample and mixed well by pipetting. 250 µL of analytical grade ethanol (Fisher Scientific, Loughborough, UK) was added and mixed well by pipetting. The entire mixture (700 µL) was added to an RNeasy Mini kit spin column placed in a 2 mL collection tube and centrifuged for 15 seconds at 10,000 x g (Model: Micro Star 17R, Manufacturer: VWR, Lutterworth, UK). Flow-through was discarded. After centrifugation, DNase treatment was performed using the RNase-free DNase set (Qiagen, Hilden, Germany). 350 µL of buffer RW1 was added directly to the column and centrifuged for 15 seconds at 10,000 x g, flow-through was discarded. DNase mixture was prepared by mixing 10 µL of DNase I stock solution with 70 µL buffer RDD. The 80 µL mixture was added to the spin column membrane and incubated at room temperature for 15 minutes. Following incubation, 350 µL buffer RW1 was added to the spin column and centrifuged for 15 seconds at 10,000 x g, flow-through was discarded. 500 µL of buffer RPE was added to the spin column and centrifuged for 15 seconds at 10,000 x g, flow-through was discarded. A further 500 µL of buffer RPE was added to the spin column and centrifuged for 15 seconds at 10,000 x g, flow-through was discarded. The spin column was transferred to a clean collection tube and centrifuged at full speed (17,000 x g) for 1 minute to fully dry the membrane. 50 µL of nuclease-free water was added directly to the spin column placed in a 1.5 mL eppendorf tube for collection. This was centrifuged for 1 minute at 10,000 x g and flow-through collected. The purity and yield of RNA was determined by absorbance

readings at 230/260/280 nm on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Loughborough, UK).

2.4: cDNA synthesis from PBMC RNA

1000 ng of total RNA, quantified on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Loughborough, UK) was used in the cDNA reaction. High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley, UK) was used for RNA extracted from human peripheral blood samples. A 2X master mix was prepared according to the table below:

Table 5: cDNA reaction mixture (High Capacity cDNA Reverse Transcription kit)

Kit Component	**Volume per reaction (µL)**
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free water	3.2
TOTAL	10

10 µL of master mix was mixed with 10 µL of sample (1000 ng) in nuclease-free water. Samples were run in a thermocycler (Model: T100, Manufacturer: BioRad) according to the following settings:

Step1: 25ºC 10 minutes

Step 2: 37ºC 120 minutes

Step 3: 85ºC 5 minutes

Step 4: 4ºC Hold

2.5: Quantitative PCR (qPCR)

qPCR reaction was run on MicroAmp Fast 96-well Reaction Plate (Applied Biosystems), on a StepOne Plus thermocycler (Applied Biosystems). Both SYBR Green chemistry and TaqMan probe methods were used.

SYBR Green: cDNA was diluted 1/50 in nuclease-free water (Sigma-Aldrich, Dorset, UK). 4.5 µL of diluted cDNA was added to 5 µL iTaq SYBR Green (Bio-Rad, Hercules, USA) and 0.5 µL of 10 mM primer (Sigma-Aldrich, Dorset, UK). Primer sequences are shown in the table below.

Table 6: List of primer sequences used

	Forward	**Reverse**
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
GATA1	CTGTCCCCAATAGTGCTTATGG	GAATAGGCTGCTGAATTGAGGG
FOG1	CGGTACTGCCGTCTTTGCA	CGTGCGAGGAGCAGTAATACTTC
NFE2	ACTCACTCATGCCCAACTCC	TCTACCGGCAAGTTGACAATC
FLI1	CGCTGAGTCAAAGAGGGACT	AATGTGTGGAATATTGGGGG
CALR	TGTCAAAGATGGTGCCAGAC	ACAACCCCGAGTATTCTCCC
CANX	ACACAGCAACCACTTCCCTT	GCCTCCGCCTCTCTCTTTAC

	Forward	**Reverse**
STAT1	ATCAGGCTCAGTCGGGGAATA	TGGTCTCGTGTTCTCTGTTCT
ISG15	CGCAGATCACCCAGAAGATCG	TTCGTCGCATTTGTCCACCA
OAS1	TGTCCAAGGTGGTAAAGGGTG	CCGGCGATTTAACTGATCCTG
MX1	AGCGGGATCGTGACCAGAT	TGACCTTGCCTCTCCACTTATC
TAP1	CTGGGGAAGTCACCCTACC	CAGAGGCTCCCGAGTTTGTG
HLA-A1	AAAAGGAGGGAGTTACACTCAGG	GCTGTGAGGGACACATCAGAG
HSPA8	ACCTACTCTTGTGTGGGTGTT	GACATAGCTTGGAGTGGTTCG
HSP90AB1	AGAAATTGCCCAACTCATGTCC	ATCAACTCCCGAAGGAAAATCTC
ITGA2B	GATGAGACCCGAAATGTAGGC	GTCTTTTCTAGGACGTTCCAGTG
TTRAP	GACAGTGAGACTCGAACACATT	CAAGGGCACAAACTCAGCAAC
DNAJA1	GACATACAGCTCGTTGAAGCA	GTGATGACGATGGTTCGGTTG
IRF1	CTGTGCGAGTGTACCGGATG	ATCCCCACATGACTTCCTCTT
DDX3X	ACGAGAGAGTTGGCAGTACAG	ATAAACCACGCAAGGACGAAC
SOCS1	TTTTCGCCCTTAGCGTGAAG	CATCCAGGTGAAAGCGGC
SOCS3	GGAGACTTCGATTCGGGACC	GAAACTTGCTGTGGGTGACC
STAT3	GAGGACTGAGCATCGAGCA	CATGTGATCTGACACCCTGAA
STAT5	TTACTGAAGATCAAGCTGGGG	TCATTGTACAGAATGTGCCGG

	Forward	**Reverse**
IRF3	TCTTCCAGCAGACCATCTCC	TGCCTCACGTAGCTCATCAC
IRF4	ATGCTTTGGAGAGGAGTTTC	CTGGATTGCTGATGTGTTC

qPCR cycle times and temperatures are shown below.

Step 1: 95ºC 30 seconds

Step 2: 95ºC 3 seconds

Step 3: 60ºC 30 seconds

Repeat Step 2 – 3 x 39

Step 4: 60ºC + 0.3ºC incremental to 95ºC 3 seconds

TaqMan: For each well, 4.5 µL of 1/50 diluted cDNA sample was added to 0.5 µL TaqMan probe and 5 µL of Fast Advanced Master Mix (Thermo Fisher) using a MicroAmp Fast-96 Reaction plate (Applied Biosystems). PCR cycle times and temperatures given below:

Step 1: 50ºC 2 minutes

Step 2: 95ºC 20 seconds

Step 3: 95ºC 1 second

Step 4: 60ºC 20 seconds

Repeat Step 3 – 4 x 39

2.6: Cell culture

Immortalised myeloid leukaemia cell lines in suspension were obtained from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmBH unless stated. Growth media used was RPMI-1640 (Invitrogen, Paisley, UK) supplemented with either 10 or 20% foetal bovine serum (Invitrogen, Paisley, UK). Cells were chosen on the basis of having an activating JAK2 mutation (HEL, SET2 and UKE1) or a previous patient MPN history (SET2 and UKE1). The first control cell line, K562, was selected as it also results in constitutive JAK/STAT pathway activation but does not carry a *JAK2* mutation. The other control, HL-60, had neither a *JAK2* activating mutation nor detectable JAK/STAT activity.

Table 7: Cell lines used in the studies

Cell line	**DSMZ #**	**Type**	**Culture conditions**	***JAK2*^{V617F} mutation status (Quentmeier *et al.*, 2006)**
K-562	ACC 10	Chronic myeloid leukaemia in blast crisis	RPMI-1640 + 10% FBS.	No
HL-60	ACC 3	Acute myeloid leukaemia	RPMI-1640 + 10% FBS.	No
HEL	ACC 11	Erythroleukaemia	RPMI-1640 + 10% FBS.	Yes - homozygous

Cell line	**DSMZ #**	**Type**	**Culture conditions**	***JAK2*^{V617F} mutation status (Quentmeier *et al.*, 2006)**
SET-2	ACC 608	Essential thrombocythemia at megakaryoblastic leukaemic transformation	RPMI-1640 + 20% FBS. Maintain between 0.2-1 x 10^6 cells/mL.	Yes - heterozygous. Both *JAK2*^{WT} and *JAK2*^{V617F}
UKE-1	Gift from Professor Anthony Green (University of Cambridge)	Essential thrombocythemia at leukaemic transformation	RPMI-1640 + 10% FBS.	Yes - homozygous

2.7: Drugs and inhibitors

Anagrelide, ruxolitinib, and givinostat (Selleckchem, Newmarket, UK) dissolved in DMSO and hydroxyurea (Sigma-Aldrich, Poole, UK) dissolved in water, were applied to cell culture at a 1/100 dilution of final volume. Fludarabine (Sigma, Poole, UK) was dissolved in DMSO to a working concentration of 100 mM.

2.8: Trypan blue exclusion assay

Cells were seeded (1 x 10^5 cells per well) in 12-well microtitre plates (Sarstedt, Leicester, UK) in 990 µL RPMI 1640 growth media + 10-20% FBS (Invitrogen, Paisley, UK). 10 µL of drug at 100 X final

concentration diluted in RPMI media was added to each well. DMSO, equivalent in dilution of highest drug concentration was added to a control well. The treated samples were mixed by gentle pipetting up and down ten times. At every 24-hour interval after treatment up to 96 hours, cells were removed fully from the wells. These were mixed by gentle vortexing and 50 µL of cell suspension was added to 50 µL 0.4% trypan blue solution (Sigma-Aldrich, Poole, UK). The mixture was incubated at room temperature for 5 minutes, then vortexed briefly, before 10 µL of the mixture was pipetted onto a haemocytometer chamber with cover slip attached. Dead cells with permeable membranes took up the dye and stained blue, live cells did not stain and reflected light. Cells were counted in each of the 16-square corner quadrants (1 mm x 1 mm x 0.1 mm). The total volume per quadrant was 0.1 µL or 1 x 10^{-4} mL. Total number of cells was divided by number of quadrants counted (4) and multiplied by the dilution factor in trypan blue (2). This gave cell count per 1 x 10^{-4} mL, final cell count per mL was determined by multiplying by 10,000. Cell viability percentage was calculated by dividing live cells by total cells (dead and live) and multiplying by 100.

2.9: 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay

Cells were seeded (2 x 10^4 cells per well) in 96-well microtitre plates (Sarstedt, Leicester, UK) with drugs or peptides in 2X or 10X dilutions. Vehicle control was added to control wells at equivalent DMSO (dimethyl sulfoxide) or water concentration to highest drug concentration. After 72 hours, four negative control wells were treated with 10 µL of a 10% Triton X-100 solution for 10 minutes. All cells were then incubated for 2 – 4 hours in the presence of 20 µL 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, USA) and phenazine methosulfate (PMS) (Sigma-Aldrich, Poole, UK). MTS/PMS mixture was prepared at a 100:5 ratio immediately prior to adding to the wells. Absorbance was measured on a microtitre plate reader (Model: SpectraMax Plus, Manufacturer: Molecular Devices, Wokingham, UK) at 490 nm.

2.10: RNA extraction on cell lines

RNA extraction was performed using the Quick RNA Mini-Prep (Zymo Research, USA). All reagents unless specified were supplied with the kit. Cell pellets were lysed in 300 µL RNA lysis using a 21G gauge needle and 1 mL syringe (Becton-Dickinson, Fraga, Spain), plunged up and down ten times. An equal volume of ethanol (Fisher Scientific, Loughborough, UK) was added to the lysate and the mixture transferred to a Zymo-Spin™ IIICG Column in a 2 mL collection tube. The spin column was centrifuged at 13,000 x g for 30 seconds and the flow through was discarded. 400 µL of RNA wash buffer was used to prewash the column (centrifuge at 13,000 x g for 30 seconds). 80 µL of DNase reaction mix (75 µL DNase digestion buffer and 5 µL DNase I) was added directly to the column membrane and incubated for 15 minutes at room temperature. After incubation, the tube was centrifuged (30 seconds at 13,000 x g). 400 µL of RNA Prep Buffer was added to the spin column and centrifuged (13,000 x g for 30 seconds). Flow-through was discarded. 700 µL of RNA Wash buffer was added to the spin column and centrifuged (13,000 x g for 30 seconds), flow-through was discarded. 400 µL RNA wash buffer was added to the spin column and centrifuged (13,000 x g for 2 minutes), flow-through was discarded. The spin column was then transferred to a clean RNase free eppendorf and 50 µL of nuclease-free water pipetted directly on to the membrane. The tube was centrifuged (13,000 x g for 30 seconds) and the spin column discarded and flow-through collected. The purity and yield of RNA was determined by absorbance readings at 230/260/280 nm on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Loughborough, UK).

2.11: cDNA synthesis from cell line RNA

Complementary DNA was synthesised from total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). Reaction mixture was as follows:

4 µL iScript reaction mix

1 µL iScript Reverse Transcriptase

1000 ng Total RNA

Nuclease-free H_2O to 20 µL

A no-reverse transcriptase control (NRT) was performed, substituting reverse transcriptase in the reaction mix for water. Nuclease-free water was used in place of RNA sample for the no-template control (NTC).

The reaction mixture (20 µL) was run in a thermocycler (Model: T100, Manufacturer: Bio-Rad, Watford, UK) per the following settings:

Step1: 25ºC 5 minutes

Step 2: 42ºC 30 minutes

Step 3: 85ºC 5 minutes

Step 4: 4ºC Hold

2.12: Cell cycle analysis

Cell fixation: Cells (K562 and HEL) at 2 x 10^5 cells/mL (5 mL total volume) were serum starved for 24 hours prior to drug treatment (1 µM anagrelide or ruxolitinib) for up to 72 hours. After incubation, media was removed and cells were washed in PBS, before being resuspended in 0.5 mL of PBS. 4.5 mL of ice-cold 70% ethanol was added while mixing to the cell suspension.

Propidium iodide (PI) staining: Cells in ethanol were centrifuged, supernatant was discarded and washed with PBS. Propidium iodide staining solution was prepared by adding 200 µL of 1 mg/mL propidium iodide (Sigma-Aldrich, Poole, UK) and 2 mg DNase-free RNase A (Sigma-Aldrich, Poole, UK) to 10 mL of a 0.1% Triton-X100 solution. 1 mL of the PI staining solution was used to stain cell pellet for 30 minutes at room temperature. PI emission was measured using a FACSVerse flow cytometer (BD Biosciences, Oxford, UK) and cell cycle analysis performed using FlowJo X software (Treestar Inc, Ashland, US).

2.13: Western blotting

Buffers used for SDS-PAGE and Western blot are given below:

Table 8: Buffer formulations for SDS-PAGE and Western blot experiments

Modified RIPA buffer	50 mM Tris (Fisher Scientific, Geel, Belgium) pH 7.5 150 mM NaCl (Fisher Scientific, Geel, Belgium) 1% NP-40 (Thermo Scientific, Rockford, USA) 10% Glycerol (Fisher Scientific, Loughborough, UK) 5 mM EDTA (Fisher Scientific, Geel, Belgium)

	Ultrapure (18.2 MΩ) H_2O (Model: Purelab Flex, Manfacturer: Elga Lab Water, High Wycombe, UK)
SDS Loading buffer	10% SDS (Fisher Scientific, Geel, Belgium) 50% Glycerol 200 mM Tris pH 6.8 Ultrapure H2O Bromophenol Blue (BDH Chemicals, Poole, UK)
SDS Running Buffer	25 mM Tris 192 mM Glycine (Melford Biolaboratories, Chelsworth, UK) 0.1% w/v SDS
Transfer buffer	25 mM Tris 192 mM Glycine 20% Methanol (Fisher Scientific, Loughborough, UK)
Tris Buffered Saline (TBS)	20 mM Tris pH 7.5 100 mM NaCl
TBS-Tween (TBS-T)	0.1% Tween-20 (Fisher Scientific, Loughborough, UK)
5% Bovine Serum Albumin (BSA) blocking solution	5% BSA (Roche, Mannheim, Germany) in 0.1% TBS-T

Primary antibody diluent (2.5% BSA)	2.5% BSA in 0.1% TBS-T

Cells were lysed in modified RIPA buffer with protease inhibitors (Roche, Mannheim, Germany) added (1 tablet per 10 mL mRIPA buffer). Total protein concentration for lysates was determined using Bradford reagent (Bio-Rad, Watford, UK) or by A280 spectrometer reading on a Nanodrop spectrophotometer (Thermo Scientific, Loughborough, UK). Lysates were loaded on a 10% SDS-PAGE gel and run at 100 – 120V until the dye front reached the end of the plate. Transfer of proteins to nitrocellulose membrane was carried out using a wet-blot apparatus with Tris/Glycine + 20% methanol transfer buffer. Transfer was carried out at +2 – 8ºC at 100V for 1 hour. Ponceau S (Sigma-Aldrich, Poole, UK) dye was used to check transfer. The nitrocellulose membrane was blocked in 5% BSA in Tris Buffered Saline + 0.1% Tween-20 (TBS-T). Primary antibody at the appropriate dilution was applied to the membrane and incubated overnight at +2 – 8ºC. Antibody dilutions are listed in Table 9 below. After incubation, the primary antibody was removed and membrane washed 5 times for 5 minutes with TBS-T. Secondary antibody, HRP-conjugated anti-rabbit IgG (Cell Signalling Technology (NEB), Hitchin, UK) at a 1/10,000 dilution in TBS was applied to the membrane and incubated at room temperature for 1 hour with gentle rocking. After incubation, the membrane was washed 5 times for 5 minutes with TBS-T followed by rinse in TBS. Enhanced chemiluminescent substrate (Thermo-Scientific, Loughborough, UK) was applied directly to the membrane and left for 1 minute before exposing to light sensitive X-ray film (FujiFilm, Tokyo, Japan) and developed using an X-ray film developer (Model: Compact X4 Manufacturer: Xograph, Stonehouse, UK)

Table 9: Antibodies and dilutions used in Western blot experiments

Antibody	**Host**	**Dilution in 2.5% BSA**	**Manufacturer**
Phospho STAT1 (Tyr701)	Rabbit	1:2000	Cell Signalling Technology

Antibody	Host	Dilution in 2.5% BSA	Manufacturer
Total STAT1	Rabbit	1:5000	Cell Signalling Technology
Phospho STAT3 (Tyr705)	Rabbit	1:1000	Cell Signalling Technology
Total STAT3	Rabbit	1:5000	Cell Signalling Technology
Phospho STAT5 (Tyr694)	Rabbit	1:1000	Cell Signalling Technology
Total STAT5	Rabbit	1:5000	Cell Signalling Technology
Phospho JAK2 (Tyr1007/1008)	Rabbit	1:2000	Cell Signalling Technology
Total JAK2	Rabbit	1:5000	Cell Signalling Technology
Monoclonal α-Tubulin	Mouse	1:10000	Sigma
GAPDH	Rabbit	1:20000	Amsbio, Abingdon, UK
HRP-conjugated anti-rabbit IgG	Goat	1:10000	Thermo Scientific
HRP-conjugated anti-rabbit IgG	Goat	1:10000	Cell Signalling Technology
HRP-conjugated anti-mouse IgG	Goat	1:10000	Thermo Scientific

2.14: Colony-forming assays

An aliquot (300 µL) of a 10 X PBMC solution (2 x 10^6 cells/mL) was added to 3 mL of MethoCult methylcellulose media, H4034 Optimum (Stemcell Technologies, Vancouver, Canada). The growth media contained recombinant human (rh) cytokines, including erythropoietin (rh-EPO) at a concentration of 0.1 U/ml. This is the concentration at which there was differential gene expression between *JAK2*^{V617F} and *JAK2*^{WT} cells (Chen *et al.*, 2010). Ruxolitinib (100 and 250 nM) was added to the

methylcellulose media at a 1/1000 dilution. The mixture was gently rolled and vortexed briefly to mix before allowing to settle. 1.1 mL of media was applied to each colony dish using a blunt end needle and syringe (Stemcell Technologies, Vancouver, Canada). Cells were incubated at 37ºC in a 5% CO_2 incubator for 14 days before colony types were identified and counted. BFU-E colonies were isolated and collected in 5 mL ice-cold PBS and centrifuged (300 x g for 3 minutes). Supernatant was discarded and pellet was resuspended in 1 mL ice-cold PBS. A cell count in 0.4% trypan blue was taken. The sample was then centrifuged at full speed (13,000 x g) in a microcentrifuge (Model: Micro Star 17R, Manufacturer: VWR, Lutterworth, UK). Supernatant was aspirated completely and the pellet stored at -80ºC.

2.15: Protein extraction for Mass spectroscopy

Lysis Buffer:

Table 10: Lysis buffer for protein extraction

20 mM HEPES	0.476 g
30 mM NaCl	0.193 g
6.4 mM Sodium Pyrophosphate	0.285 g
1 mM Sodium Orthovanidate	0.018 g
	Made up to 100 mL with H_2O and pH to 8.5 with 1M NaOH

Urea (Sigma-Aldrich, Poole, UK) was weighed (4.8 g) in a 50 mL tube and 7 mL of lysis buffer was added to dissolve. pH was checked to ensure it was still 8.5. Mixture was filter sterilised through 0.22 µm syringe filter (Sartorius). Phosphatase Inhibitor Cocktail II and III (Sigma-Aldrich, Poole, UK) were added at a 1/100 dilution. 20 µL Benzonase® Nuclease (Sigma-Aldrich, Poole, UK) was added to the mixture. 50 µL was added to each frozen lysate and cells sheared using 21G needle and 1 mL syringe (Becton-Dickinson, Fraga, Spain). Lysed cells were centrifuged for 15 minutes at 15,000 RPM at 4ºC.

Total protein concentration was determined using Quick-Start Bradford dye reagent (Bio-Rad, Hercules, USA). Sample was diluted 1/200 with lysis buffer and 90 µL loaded on a microtitre plate. 90 µL Bradford dye reagent was added and absorbance at 595 nm was measured using a microtitre plate reader. A standard curve was generated using BSA standards (Bio-Rad, Hercules, USA). Protein concentrations were equalised in 50 µL total volume. 500 mM DTT was prepared by adding 0.0385 g DTT in 500 µL water. 0.5 µL of the DTT solution was added to each tube to give a final 5 mM

concentration. Tubes were incubated for 30 minutes at room temperature. 1.56 µL of 500 mM iodoacetamide was added to each sample tube and incubated in the dark for 20 minutes.

Lys-C Protease (Thermo-Scientific, Rockford, USA) was prepared in 20 mM HEPES buffer (pH: 8.5) to a final concentration of 0.1 µg/µL. This was added at a 1:100 ratio to total protein in each tube. 0.2 µg Lys-C was added to tube containing 20 µg protein (2 µL of 0.1 µg/µL solution). This was incubated for 4 hours at 37ºC with shaking. After incubation, samples were diluted to final concentration of 2 M urea with 20 mM HEPES pH 8.5 buffer. 0.5 µL of 100 mM $CaCl_2$ was added to each tube. Trypsin protease (Thermo Scientific, Rockford, USA) was added at a 1:20 ratio with total protein – 1 µg per tube. Samples were incubated on shaking platform overnight at 37ºC.

Isobaric tagging and relative quantification (iTRAQ) reagent labelling:

Each iTRAQ reagent vial was reconstituted by the addition of 50 µl of isopropanol at room temperature. The vials were vortexed and centrifuged briefly. The contents of each of the vials was added to sample tube containing 100 µg of digested protein.

Samples were acidified to 1% with trifluoroacetic acid (TFA) and centrifuged for 20 minutes at 10,000 x g at 4ºC. The supernatant was removed and retained. 2 x 2 mL of 80% acetonitrile/0.5% acetic acid was slowly pushed through a tC_{18} SPE 3cc cartridge (Waters, Elstree, UK). This was followed by 2 x 2 mL methanol. 2 x 2 mL 0.1% TFA was then pushed through the column. Sample was added to the column and allowed to flow through by gravity. After 20 minutes, any remaining sample in the column was gently pushed through. 2 x 2 mL of 80% acetonitrile/0.5% acetic acid was gently pushed through. Aliquots were lyophilised for storage and mass spectroscopy preparation.

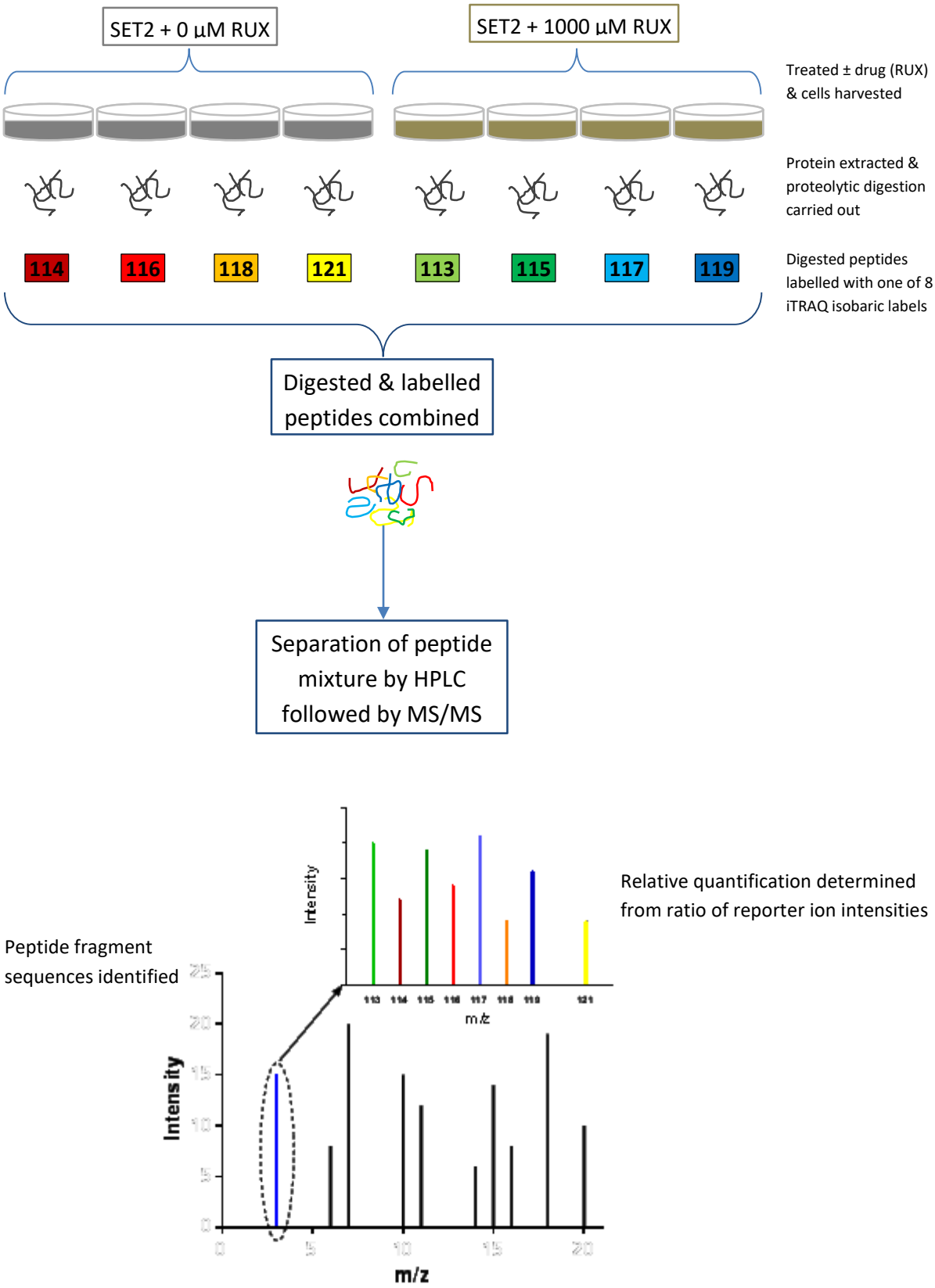


Figure 11: Workflow for iTRAQ labelling and quantification of differentially expressed proteins in response to ruxolitinib treatment.

2.16: Mass Spectroscopy

Workflow for labelling and quantification is shown in Figure 11. Sample loading and fractionation was performed using an Eksigent Ekspert NanoLC 400 UHPLC. Peptides were eluted from the reverse phase C18 nano-column using a flow rate of 300 nL/min. Mass spectroscopy was carried out on a TripleTOF 6600 MS/MS instrument with electrospray ionisiation (AB SCIEX, Framingham, MA, USA). The instrument was operated in data dependent mode. Peptides with multiple charges (2^+ to 4^+) with a charge to weight ratio (*m/z*) between 400 and 1600 were selected for MS/MS. Visualisation of altered biological processes was performed using these differentially expressed proteins and Cytoscape v3.5.0 (Shannon *et al.*, 2003) with ClueGO plugin (Bindea *et al.*, 2009) with gene ontology terms. Interactions between the proteins were examined and demonstrated using the web program STRING (version 10.5) (Szklarczyk *et al.*, 2015).

2.17: Statistics and data analysis

IC_{50} values from MTS assays were determined, where possible, using GraphPad Prism software v6.05. A log transformation of the concentration values was performed and a three-parameter non-linear curve fit applied. IC_{50} was determined as the concentration at which 50% maximal inhibition occurred on the curve fit. Each MTS assay was carried out on three separate occasions.

Relative gene expression was calculated using the $\Delta\Delta C_T$ method, where *GAPDH* was used as the control gene. Sample was cDNA derived from PBMCs from ET patients and control sample was PBMC cDNA from a healthy volunteer. C_T values were acquired from the StepOne instrument software (v2.3).

$$\mathbf{\Delta C_T} = C_{T(\text{Gene of Interest})} - C_{T(\text{GAPDH})}$$

$$\mathbf{\Delta\Delta C_T} = \Delta C_{T(\text{Sample})} - (\text{Average } \Delta C_{T(\text{Control Sample})})$$

Relative quantification given by following transformation;

$$RQ = 2^{-(\Delta\Delta Ct)}$$

Median gene expression of the 36 ET samples was compared against that of 7 healthy volunteers. Significance was determined using the Mann-Whitney U-test. Kruskal-Wallis with Dunn's Multiple Comparison test was used to calculate significance for an independent variable between three or more groups. All tests were carried out using GraphPad Prism v6.05. Significance stars are given as follows:

(*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; (****) $p < 0.0001$

A post-hoc power analysis using G*Power (v 3.1.9.2) for Windows was performed for genes that were significantly up or downregulated in ET patient samples. Power values for *GATA1, FLI1, CALR,* and *CANX* were 0.89, 1.00, 0.99, and 0.58 respectively.

Overall changes to proportions of cells in each stage of the cell cycle were determined using the chi-square test in GraphPad Prism. In individual cell cycle stages, a t-test (α-level = 0.05) was used to calculate whether treatment had any significant effect. Gating of cells was done using FlowJo software v 10.

Gene expression changes in cell lines were calculated as described for ET patient derived samples. A t-test (α-= 0.05) (GraphPad Prism) was used to determine significance.

Output files from the mass spectroscopy instrument were processed with Protein Pilot software (version 5.0.1) using Paragon™ Algorithm (version 5.0.1.0, 4874). A thorough search was performed against human database, Human_NR_UP000005640_LastMod20Dec15.fasta. The resulting data file was exported to Microsoft Excel for data analysis. Proteins were filtered by unused protein score, where a score ≥ 1.3 corresponded to a confidence level of 95%. Proteins with an unused protein score < 1.3 were excluded from the analysis. Ratios between control labels (114, 116, 118, and 121) were compared against treated labels (113, 115, 117, and 119) and those less than 0.8 were selected. At

least two spectra, with p-values less than 0.05, were required for identification and relative quantification.

Percentages of ruxolitinib treated cells in colony forming assay dishes were calculated relative to vehicle control (DMSO) treated samples. An independent t-test ($\alpha = 0.05$) was used to show significance between untreated and treated colonies. This was carried out for four PV samples, four ET samples and a healthy control in triplicate.

CHAPTER 3: *GATA1* expression levels in the peripheral blood of patients with essential thrombocythaemia

3.1: Introduction

Essential thrombocythaemia (ET) is characterised by an elevated circulating platelet count (> 450 x 10^9/L) and increased numbers of megakaryocytes (Arber *et al.*, 2016). Estimated prevalence rates in the UK are 18 per 100,000 (Titmarsh *et al.*, 2014). Overall median survival (19.8 years) is longer in ET than seen in PV (13.5 years) but still lower than the age-matched control population (Tefferi *et al.*, 2014a). Reflecting the longer survival rates, the risk of disease transformation, myeloid or leukaemic, is low in the first decade after diagnosis (9.1% and 1.4% respectively) although it increases considerably into the second (28.3% and 8.1%), and third decades after diagnosis (58.5% and 24.0%) (Wolanskyj *et al.*, 2006).

Treatment of ET is aimed at reducing thrombotic events and leukaemic transformation (O'Sullivan and Harrison, 2017). It is based on current symptoms and risk factors, including age, platelet count, and the occurrence of previous thrombotic events (Beer *et al.*, 2011). Patients with a history of previous thrombotic complications, very high platelet counts (> 1500 x 10^9/L) or of increased age (> 60 years old) are considered high risk, where cytoreductive therapy (hydroxyurea) is recommended to control platelet counts (Alimam *et al.*, 2015). Aspirin is used in conjunction with hydroxyurea therapy or on its own in lower risk patients (Tefferi and Barbui, 2015). In high risk patients who are resistant to hydroxyurea, anagrelide is used as a second-line therapy. Anagrelide is also used concomitantly with hydroxyurea as a method to avoid dose limiting toxicities encountered with a single therapy or to overcome poor efficacy (Gugliotta *et al.*, 2014). In pregnant women, treatment with hydroxyurea is avoided due to its teratogenicity in non-human mammals (Beer *et al.*, 2011). In these cases, aspirin is recommended unless contraindicated, or interferon-α if a cytoreductive agent is needed (Beer *et al.*, 2011, Finazzi, 2012).

Mutations in ET patients are found in *JAK2*^{V617F} (Baxter *et al.*, 2005, James *et al.*, 2005, Kralovics *et al.*, 2005, Levine *et al.*, 2005) *MPL*$^{W515L/K}$ (Tefferi, 2010, Vainchenker *et al.*, 2011), and several in the gene encoding calreticulin (*CALR*) (Klampfl *et al.*, 2013, Nangalia *et al.*, 2013). Both *JAK2* and *MPL* mutations

are “gain of function”, and result in constitutive activation of the JAK/STAT signalling pathway (Chaligné *et al.*, 2008). Calreticulin is a protein located in the endoplasmic reticulin (ER) which sequesters Ca^{2+} (Luo and Lee, 2013). Additionally, it plays a role in post-translational modifications of proteins in the ER, recognising N-linked glycans on glycoproteins and processing them for further folding or degradation (Michalak *et al.*, 2009, van Leeuwen and Kearse, 1996). Mutations in calreticulin have been identified in 33% of ET and MF patients (Andrikovics *et al.*, 2014).

GATA1 is a transcription factor with a critical role in haematopoiesis, specifically in erythrocyte and megakaryocyte development, as well as in eosinophil and mast cell lineages (Ferreira *et al.*, 2005). The structure is composed of three functional domains, two zinc fingers and an N-terminal activational domain. One of the zinc fingers (C) is responsible for binding to the consensus sequence on the DNA (Crispino, 2005). The other zinc finger (N) also has DNA binding activity, specific to a palindromic consensus sequence, as well as being important for the recruitment and binding of a co-factor, friend of GATA1, (FOG1) (Tsang *et al.*, 1997).

The FLI-1 (friend leukaemia integration 1) transcription factor is required for normal megakaryocyte development. It binds to Ets promoters on megakaryocyte specific genes, including *GPIX, cMPL* and *αIIb* (Pang *et al.*, 2006). Knockout of FLI1 results in moderately reduced levels of *cMPL* and *αIIb,* and a dramatic reduction in the late stage gene, *GPIX* (Hart *et al.*, 2000). FLI-1 can also associate with GATA1 and FOG1 elements to provide a synergistic effect on gene expression (Pang *et al.*, 2006).

Nuclear factor erythroid-2 (NFE2) is a haematopoietic transcription factor and is essential for normal platelet formation (Shivdasani *et al.*, 1995). Overexpression of *NFE2* has been found in the vast majority of PV patients (Goerttler *et al.*, 2005). Increased *NFE2* is also responsible for delayed erythroid maturation and results in an increase in the number of mature erythroid cells deriving from a single progenitor (Mutschler *et al.*, 2009).

3.2: Aims

This study will examine expression of *GATA1*, along with critical haematopoietic genes involved in both early and late stage megakaryopoiesis. It will also investigate whether changes in the levels of these genes correlate to each other as well as to key clinical and haematological parameters. Finally, the related ER chaperone proteins, calreticulin and calnexin, will be studied to determine whether their expression is linked to mutational status or haematological markers of disease. Correlation between the two may also indicate their common role in the ER is an important factor in the pathogenesis of ET.

The relative expression levels of these transcription levels in ET patients and their correlation to clinicopathological features will hopefully provide an insight into the molecular mechanisms of MPN as well as determining their suitability as diagnostic and prognostic markers of disease.

3.3: Methods

Methods are described in brief here; for full methodology refer to Chapter 2. Mononuclear cells were isolated from the peripheral blood of ET patients (n = 36) and healthy donors (n = 7) using Ficoll-Paque separation gradient. Total RNA was obtained using TRIzol®/chloroform extraction techniques. Complimentary DNA (cDNA) was synthesised using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was run using either Quantinova Sybr Green dye (Qiagen, Manchester, UK) or Taqman probes (Life Technologies, Paisley, UK).

3.4: Results

3.4.1: Essential thrombocythaemia clinical parameters

Clinical and haematological parameters for patients enrolled in this study are listed in Table 11. Age, sex, platelet count, mutation type (if present) and current therapy were recorded for each patient. Platelet counts were measured at two points, firstly at the time of essential thrombocythaemia (ET) diagnosis and secondly when a peripheral blood sample was drawn for analysis in this study. The total population median age was 68.5 years with female patients slightly younger (median age: 65.5 years) compared to males (median age: 70 years). There were also more female than male patients enrolled (1.6:1) in the study. 47% of patients carried a mutation in *JAK2,* 11% had a *CALR* mutation and 38% had no identified mutation in *JAK2*, *CALR* or *MPL* (triple-negative). One single patient had a mutation in the gene encoding the thrombopoietin receptor, *MPL*. Treatment regimens at the time of sampling were also recorded. 53% of patients were being treated with the cytoreductive agent hydroxyurea, 14% were on the platelet-lowering drug anagrelide. 17% of patients were treated with a combination of the two therapies, while the remainder of patients (17%) had their condition monitored and were not undergoing any drug treatment.

Table 11: ET patient cohort clinical data

	TOTAL	MALE	FEMALE
Number of patients	36	14	22
Age (MEDIAN/RANGE)	68.5 (36-92)	70 (59-81)	65.5 (36-92)
Platelet count (MEDIAN/RANGE) x10^9/L	444 (190-758)	462.5 (304-600)	419 (190-758)
Platelet count (diagnosis) (MEDIAN/RANGE) x10^9/L	706 (449-1972)	740 (552-1972)	681.5 (449-956)
Mutation			
JAK2	17	7	10
MPL	1	1	0
CALR	4	1	3
Triple Negative	14	5	9
Treatment			
ANA	5	1	4
HU	19	8	11
ANA+HU	6	4	2
WW	6	1	5

ANA: anagrelide. HU: Hydroxyurea. WW: Watch & Wait

3.4.2: *GATA1* is significantly upregulated in the peripheral blood of ET patients with a moderate negative correlation to platelet counts

Transcriptome analysis of RNA isolated from the mononuclear cells of ET patients against healthy donor controls showed that the key haematopoietic transcription factor, *GATA1,* was significantly upregulated ($p < 0.05$) in ET patients (n = 36) compared to healthy controls (n = 7) (Figure 12). Expression of *GATA1* was found to be unrelated to the choice of therapy (Figure 13), which in our cohort of patients included anagrelide (ANA), hydroxyurea (HU), a combination of anagrelide and hydroxyurea, and those currently not being treated (WW). A moderate significant negative correlation ($r = -0.4129$, $p < 0.05$) was found between measured platelet level and *GATA1* expression at the time the sample was taken. Samples were separated into three equal sized bins corresponding to measured platelet counts (Figure 14), Low: 190 – 373, Med: 375 – 500, High: 508 – 758 x 10^9 /L. *GATA1* expression in the medium and high platelet groups were significantly lower than observed in the low platelet group ($p < 0.05$). These results suggest that raised *GATA1* expression is a marker for ET, although it does not positively correlate with measured platelet counts in patients undergoing cytoreductive or platelet reducing therapies.

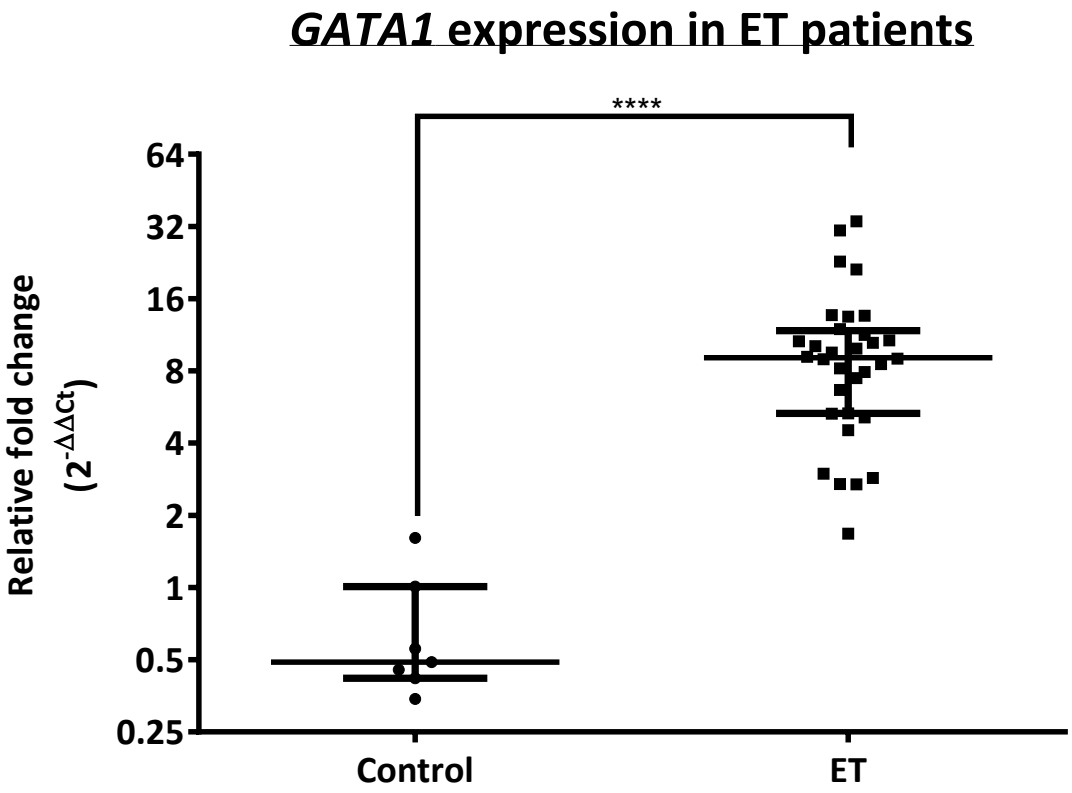


Figure 12: Median *GATA1* expression in the peripheral blood mononuclear cells of ET patients versus healthy controls. *GATA1* is significantly upregulated in the peripheral blood of ET patients (p = 0.0003 – Mann Whitney U Test).

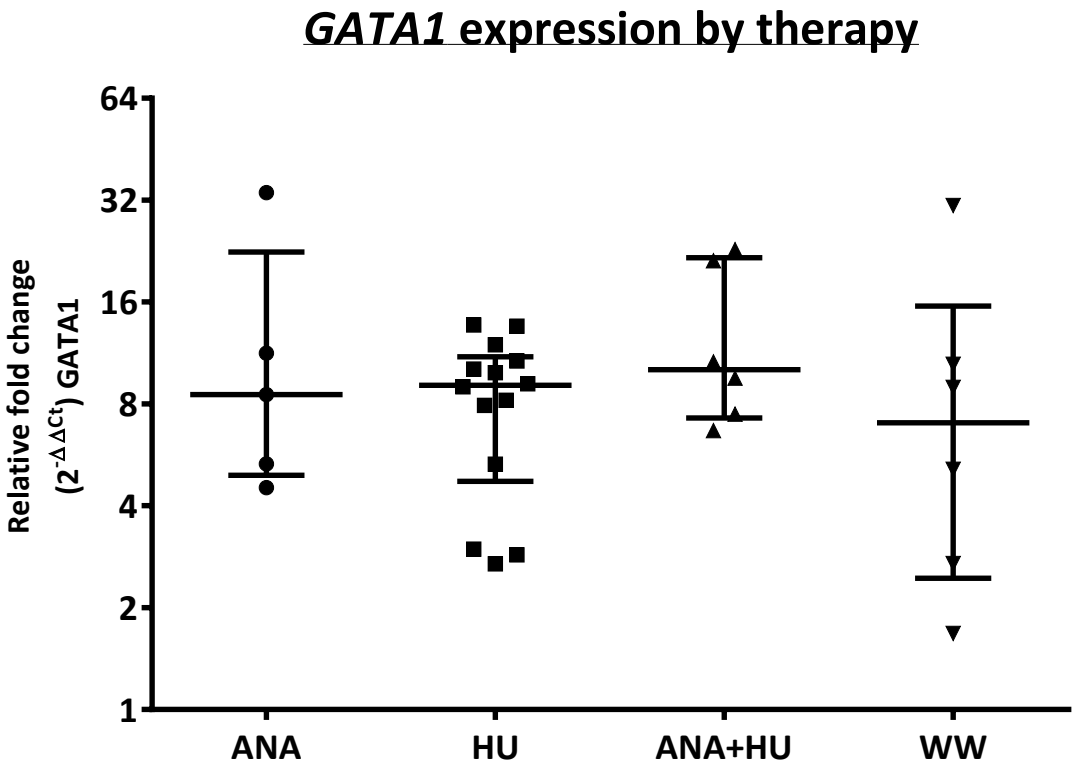


Figure 13: Median *GATA1* expression in ET patients by current therapy. ANA: anagrelide, HU: hydroxyurea, WW: Watch and wait (no treatment). *GATA1* expression was not affected by choice of therapy (Dunn's Multiple Comparison Test with Kruskal-Wallis test; $p = 0.6183$).

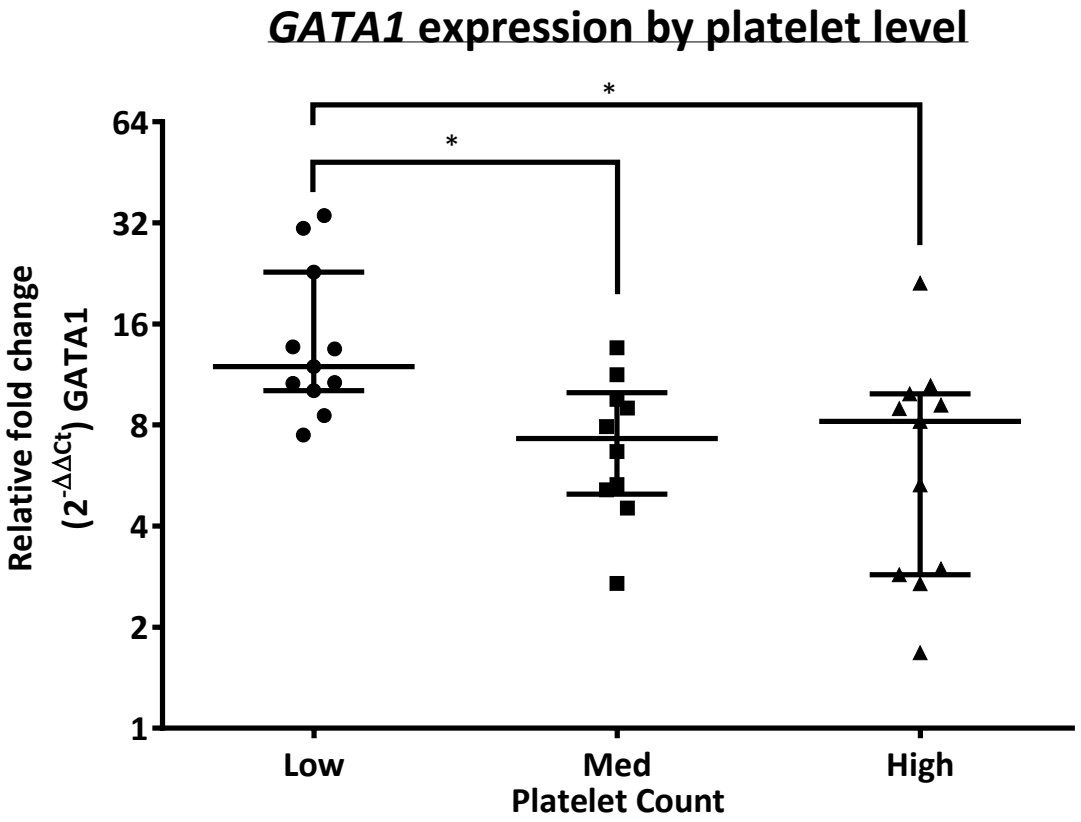


Figure 14: Median *GATA1* expression plotted against platelet counts. Platelet counts were separated into equal sized bins (Low: 190 – 373, Med: 375 – 500, High: 508 – 758 x 10^9 /L) *GATA1* was significantly raised in patients (Dunn's Multiple Comparison Test with Kruskal-Wallis test; p = 0.0059).

3.4.3: *GATA1* upregulation was independent from changes in *FLI1* and *NFE2* expression

NFE2, which is a direct transcriptional target of *GATA1,* did not shown any change in median expression (p = 0.1310) relative to healthy control samples (Figure 15). This does not follow the expected gene expression pattern given the significant increase in *GATA1* observed in Figure 12. Platelet counts showed a moderate positive correlation (r = 0.5075; p = 0.0019) with expression levels of *NFE2* in ET patients (Figure 16). Therefore, higher levels of *NFE2* were associated with an increased number of circulating platelets. This is contrary to what was observed in Figure 14, where increased *GATA1* expression correlated with lower platelet counts. From this we can conclude that the rise in *GATA1* expression seen in ET patients does not directly result in the upregulation of one of its key megakaryopoietic gene targets, *NFE2.*

In contrast to *GATA1* over-expression in ET patients, *FLI1* was significantly downregulated ($p < 0.0001$) in the PBMCs of ET patients compared to healthy controls (Figure 17). This corresponded to a nine-fold decrease in relative *FLI1* expression. Unlike *NFE2* and *GATA1,* there was no direct correlation between platelet levels and the reduced *FLI1* expression in ET patients (r = 0.2332; p = 0.1710) (Figure 18). The reduction of *FLI1,* critical for normal megakaryopoiesis, in PBMCs does not appear to negatively impact platelet production in these patients. It is not known whether this decrease could contribute to haematopoietic clonal cell expansion, a key characteristic of ET and other MPNs.

These results suggest that changes in *GATA1* expression observed in PBMCs do not directly affect the levels of other key haematopoietic transcription factors involved in both early and late megakaryopoiesis, *FLI1* and *NFE2*.

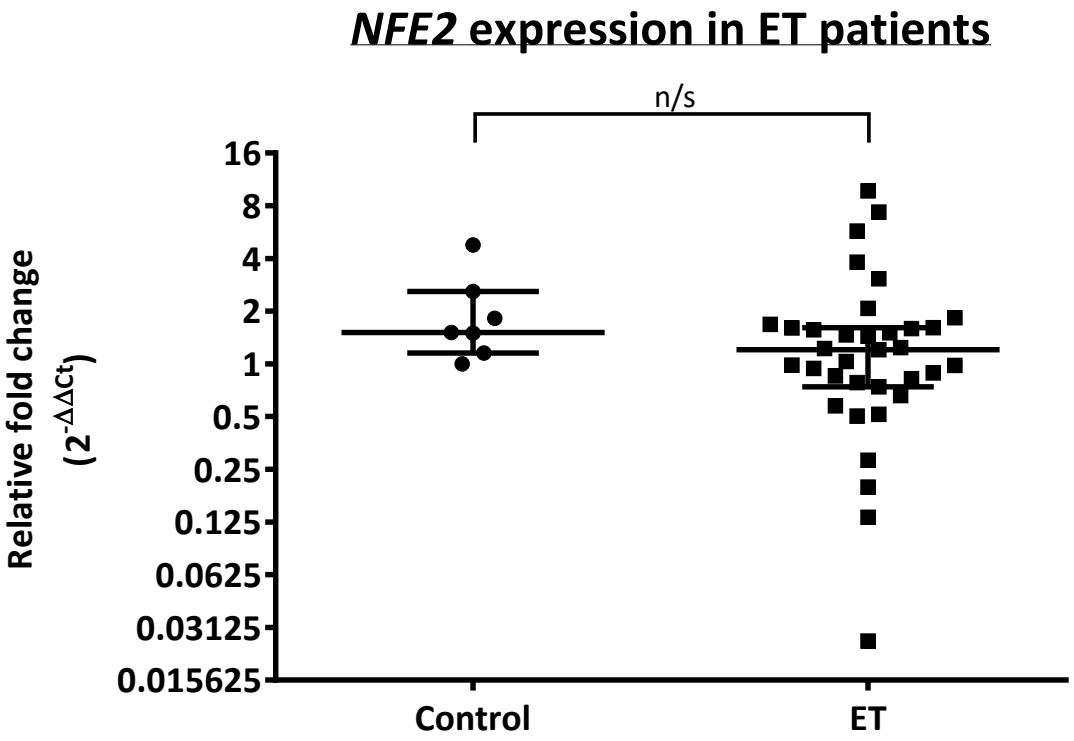


Figure 15: Expression of the haematopoietic transcription factor *NFE2* in ET patients relative to healthy controls. No significant difference between the median values in ET and controls was observed (p = 0.1310 – Mann Whitney U Test).

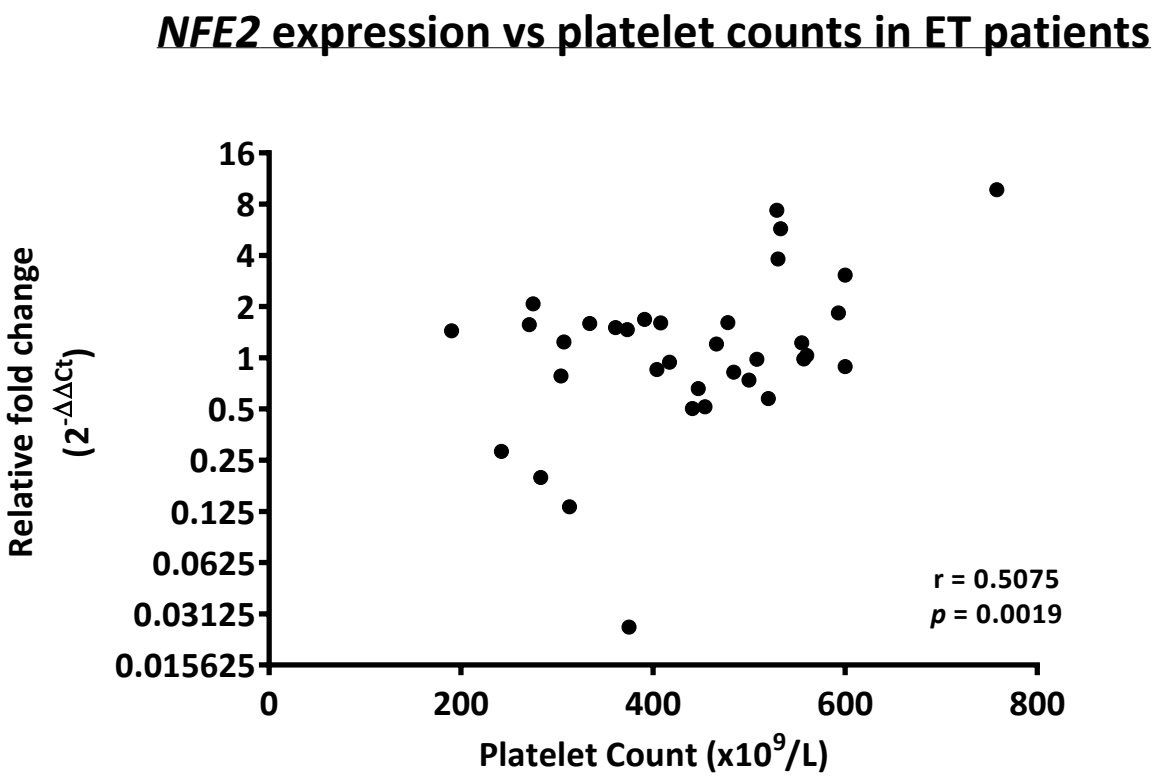


Figure 16: *NFE2* expression plotted against measured platelet counts. A moderate positive correlation was observed, $r = 0.5075$, and this was significant, $p = 0.0019$.

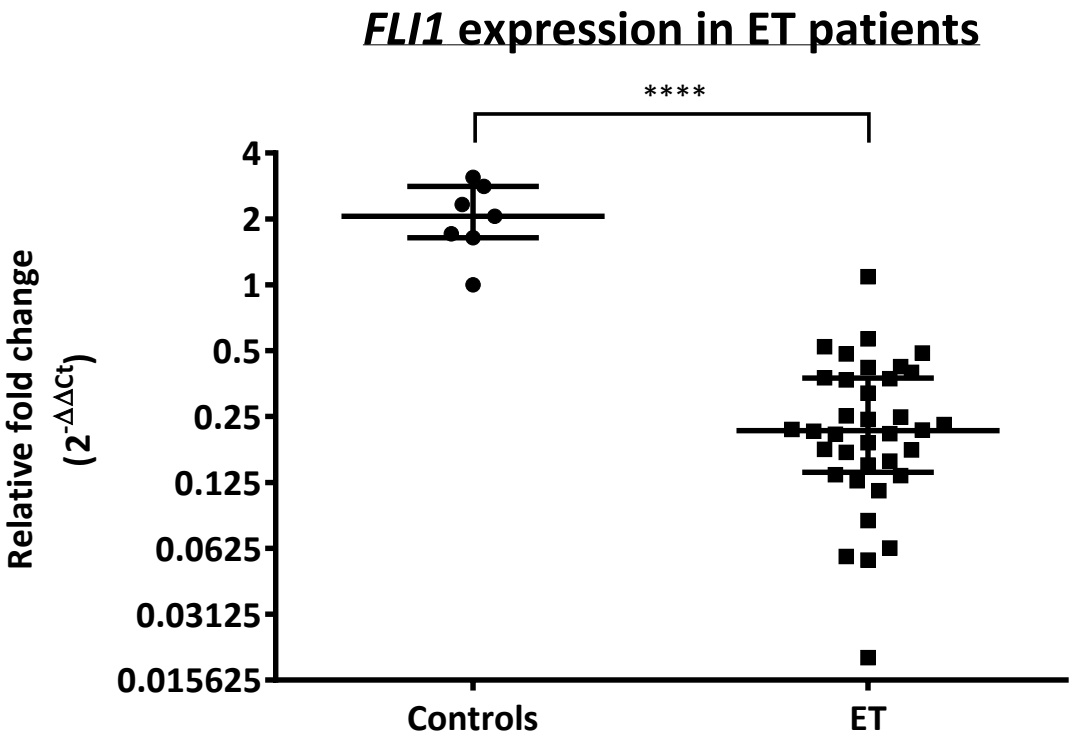


Figure 17: Median expression of the haematopoietic transcription factor *FLI1* in ET patients relative to healthy controls. Levels of *FLI1* were significantly lower in ET patients versus controls ($p < 0.0001$ – Mann Whitney U Test).

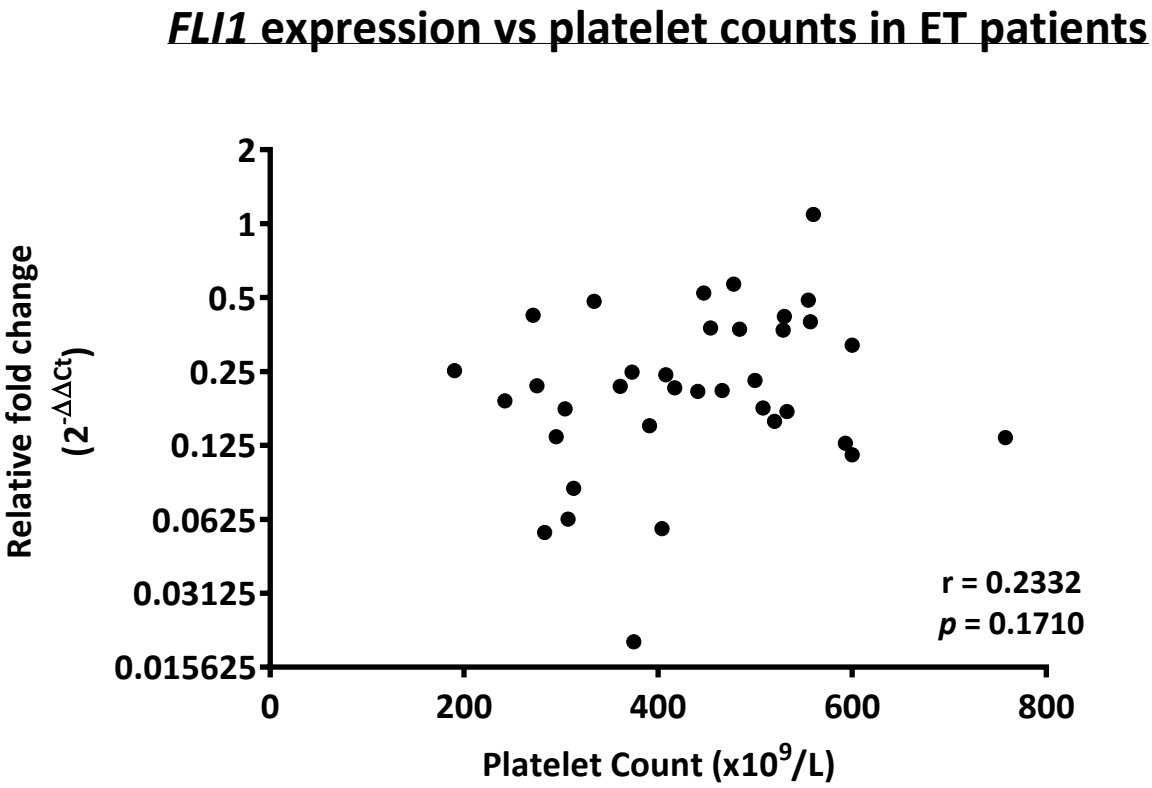


Figure 18: *FLI1* expression in ET patients plotted against measured platelet counts. No significant correlation was observed (r = 0.2332; p = 0.1710).

3.4.4: *CALR* and *CANX* are significantly downregulated in the peripheral blood of ET patients and levels of *CALR* are higher in *JAK2* mutated patients

Expression of the gene for the endoplasmic reticulum chaperone protein, calreticulin, was lower in ET patients compared to healthy controls (Figure 19). A threefold decrease in relative *CALR* expression for ET patient samples was observed which was significant ($p < 0.05$; Mann-Whitney U test). *JAK2* mutations (exon 12 and $JAK2^{V617F}$) were associated with a greater than twofold increase in relative *CALR* levels (median: 0.591) than those found in triple negative patients (median: 0.2045) (Figure 20); although no significant differences were observed between the *CALR* mutated and either *JAK2* or triple-negative groups (Figure 20). Therapy choice that patients were on at the time of sampling did not have any significant effect ($p > 0.05$) on relative *CALR* expression (Figure 21).

Gene expression for the related chaperone protein, calnexin (*CANX*), was also downregulated ($p < 0.05$ – Mann-Whitney U test). There was a fivefold decrease in relative *CANX* expression in the ET patient group (median *CANX* expression: 0.2725 versus 1.350) (Figure 22). There was a significant moderate correlation ($r = 0.5137$; $p < 0.05$) between *CALR* and *CANX* expression in ET patients (Figure 23). The reduction of both these ER chaperone proteins, and the correlation between the two, may indicate that their shared role may be a factor in the pathogenesis of the disease.

It was also shown that triple-negative patients had higher platelet counts than either $JAK2^{V617F}$ or *CALR* mutated, although the difference was not significant (Dunn's Multiple Comparison Test with Kruskal-Wallis test; $p > 0.05$). These results may be partly due to the low number of samples, particularly in the *CALR* mutated group.

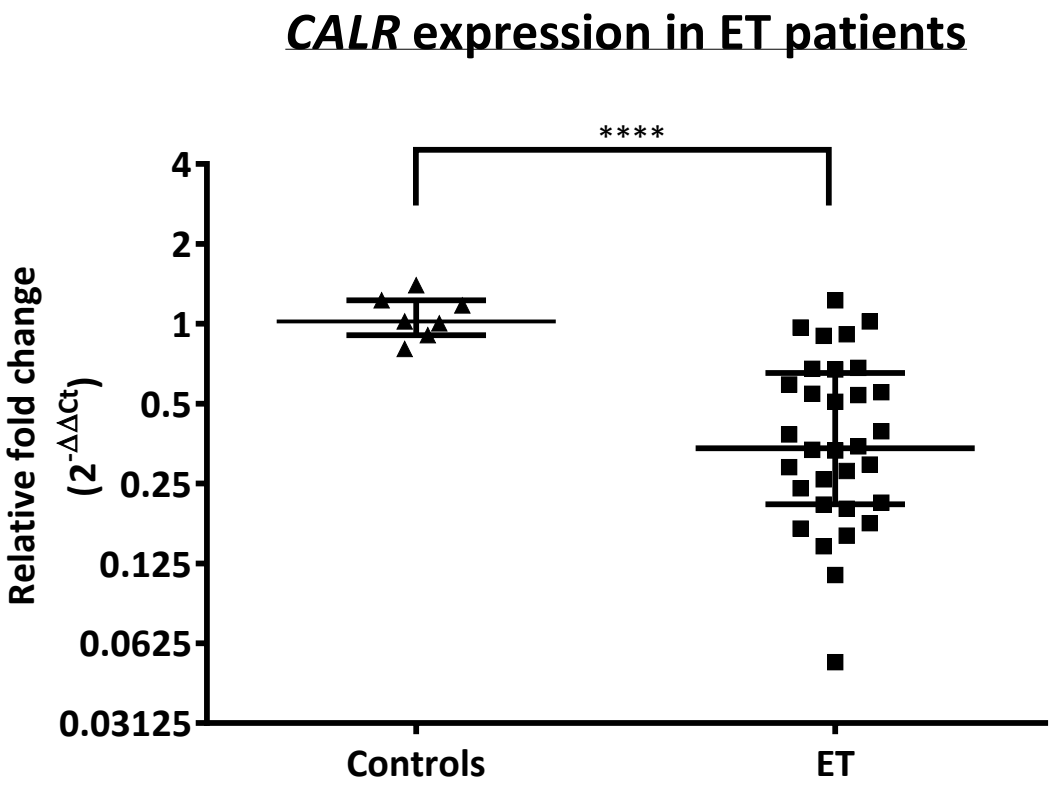


Figure 19: Median *CALR* expression in ET patient samples versus healthy controls. *CALR* was significantly downregulated in ET patient samples ($p < 0.05$ – Mann-Whitney U test).

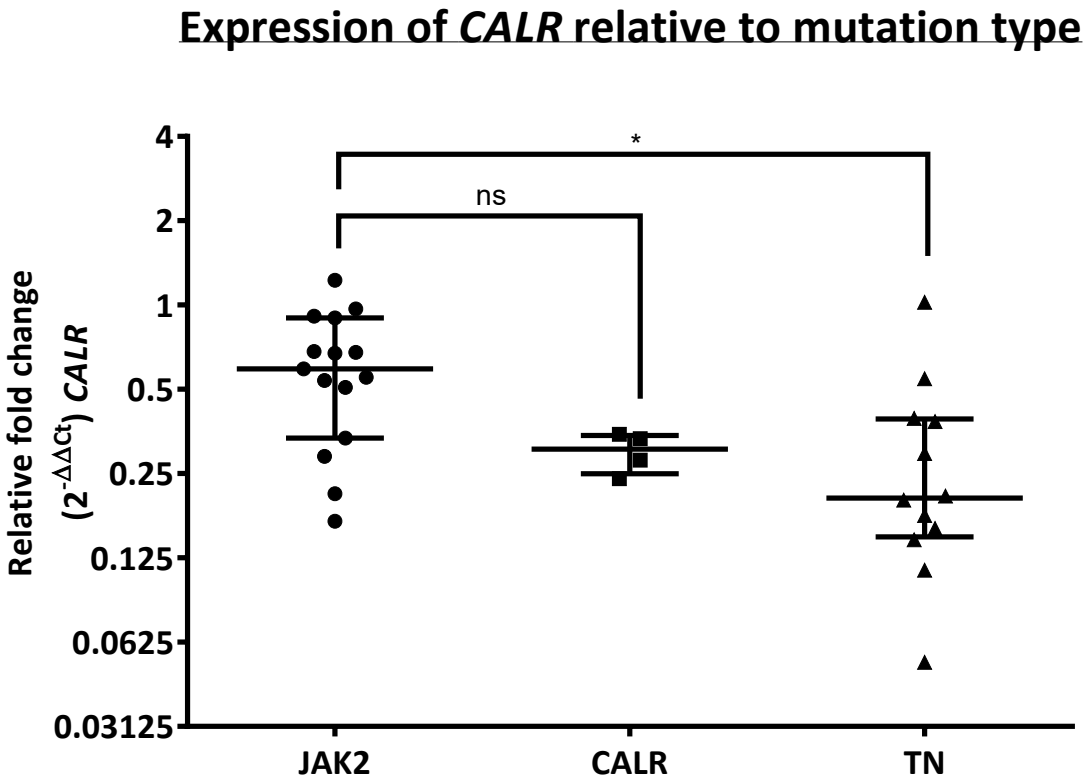


Figure 20: Calreticulin expression by recorded mutation type (*JAK2*, *CALR*, or triple-negative) in ET patients. Higher levels of *CALR* were observed in *JAK2* mutated versus TN patients (Dunn's Multiple Comparison Test with Kruskal-Wallis test; $p < 0.05$).

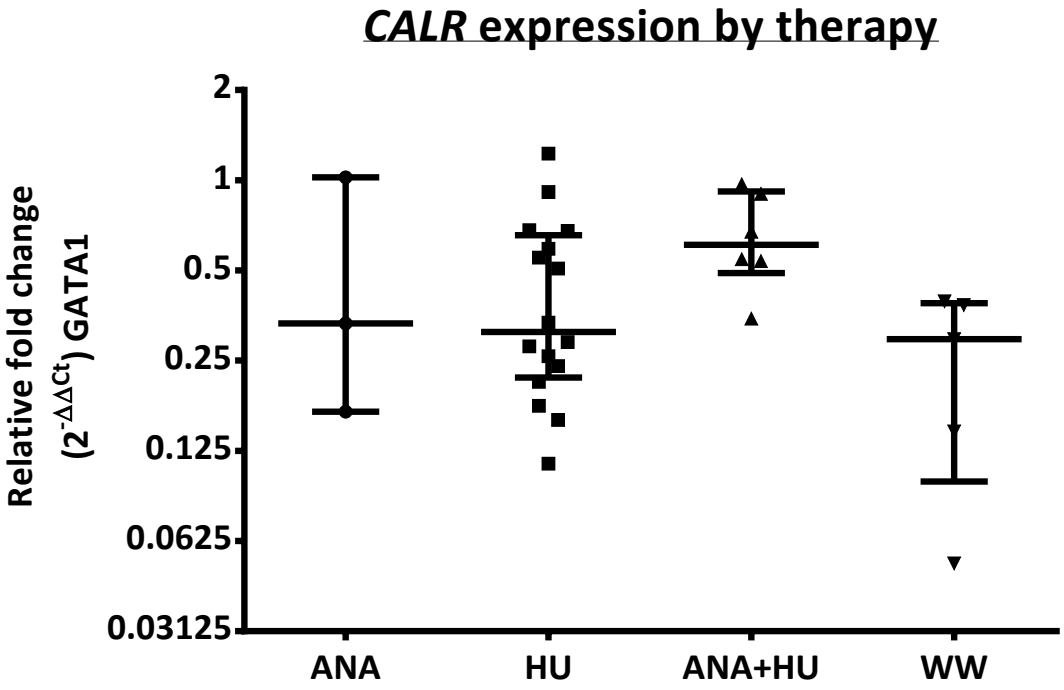


Figure 21: Expression of *CALR* by current treatment in ET patients. Treatments were anagrelide (ANA), hydroxyurea (HU), combination ANA + HU, or watch and wait/no treatment (WW). Choice of therapy or combination did not significantly affect *CALR* levels (Dunn's Multiple Comparison Test with Kruskal-Wallis test; $p > 0.05$).

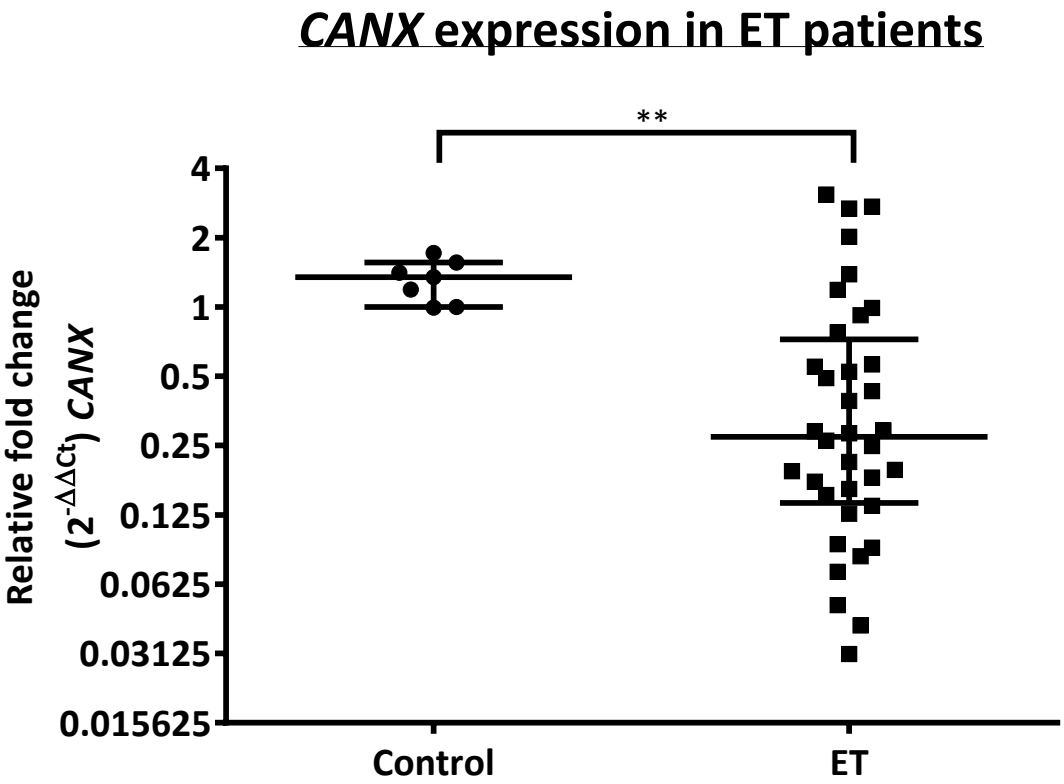


Figure 22: Calnexin expression in ET patients versus controls. The median level of *CANX* was significantly lower in ET patients ($p < 0.05$; Mann-Whitney U test).

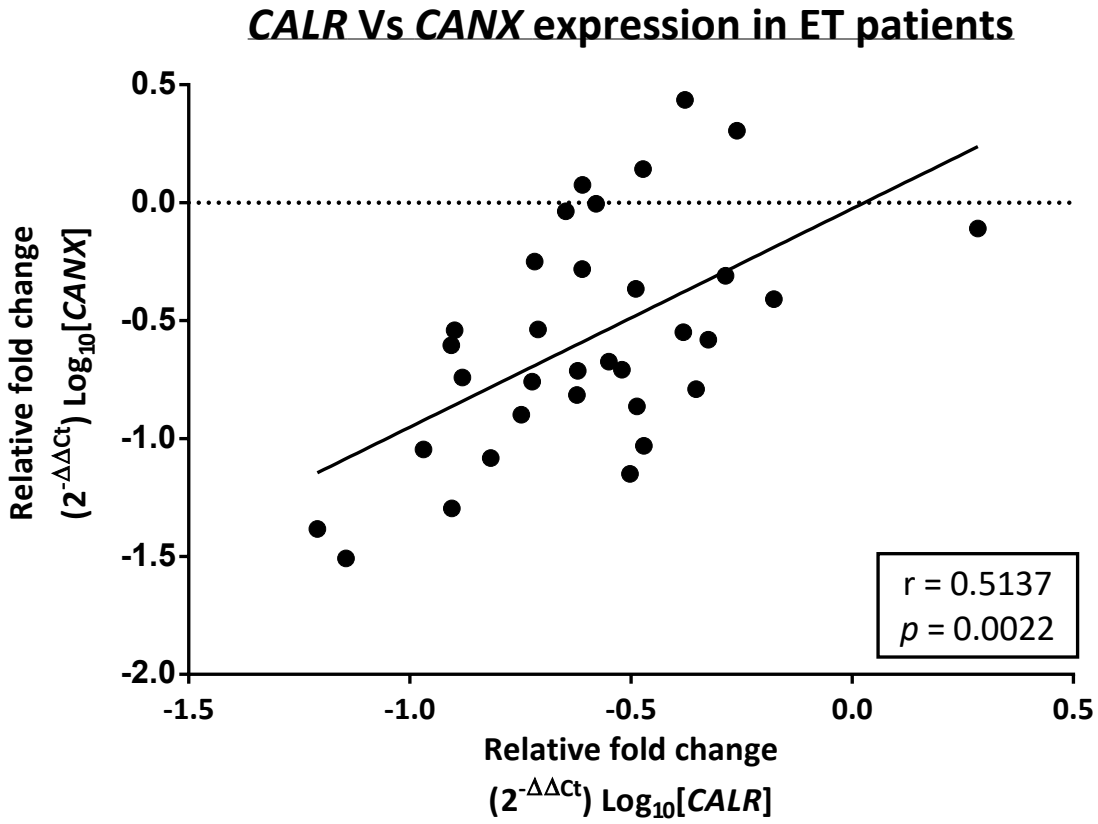


Figure 23: Correlation of *CANX* expression to *CALR* in ET patients. Levels of calnexin were moderately correlated (r = 0.5137) with those of *CALR* and this was significant ($p < 0.05$).

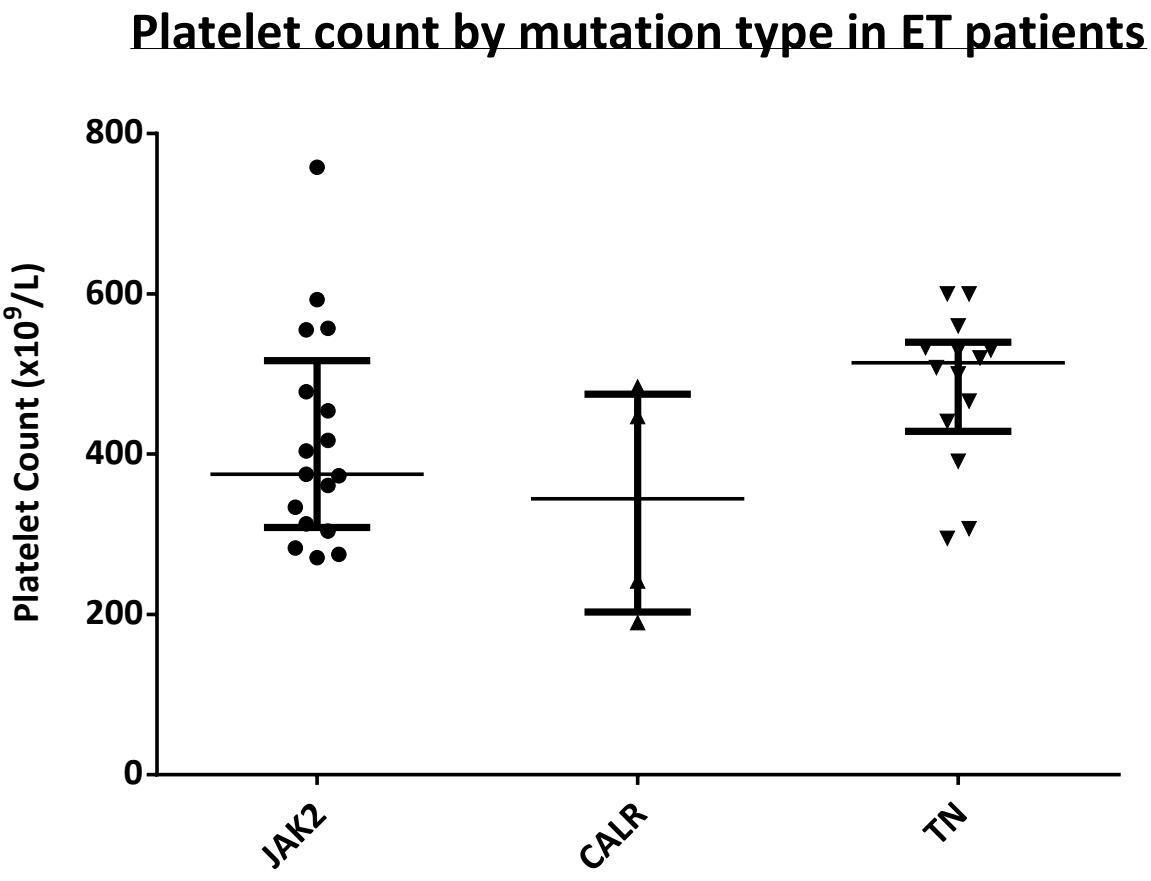


Figure 24: Platelet counts plotted by recorded mutation in ET patients (*JAK2*, *CALR* or triple-negative). Mutational status did not significantly affect platelet levels ($p > 0.05$; Dunn's Multiple Comparison Test with Kruskal-Wallis test). One sample positive for a *MPL* mutation (platelet count: 557 x 10^9 /L) was omitted from the analysis.

3.5: Discussion

The clinical and haematological parameters for our cohort of patients showed the expected higher female to male ratio as previously reported in other studies (Brière, 2007, Mesa *et al.*, 1999), as well as the increased prevalence in younger females (Brière, 2007, Wolanskyj *et al.*, 2006). The presence of a *JAK2* mutation in these ET patients reflected the frequencies in the literature, where *JAK2*^{V617F} mutations were found in 50 – 60% of ET patients (Alimam *et al.*, 2015); however there was a lower than expected number of patients carrying a *CALR* mutation (11%) compared to other published reports (24 – 32%) (Klampfl *et al.*, 2013, Nangalia *et al.*, 2013, Rumi *et al.*, 2014a, Tefferi *et al.*, 2014b). In addition, there were a high number of triple-negative patients in our samples (carrying no known mutations in *JAK2*, *MPL* or *CALR*), 38%, versus expected 12 – 15% (Tefferi *et al.*, 2014c, Tefferi *et al.*, 2014b, Nangalia *et al.*, 2013). The small cohort size may be responsible for the discrepancies observed between the frequencies of *CALR* and triple-negative patients and those reported in the literature rather than any localised population aspect. It is also noted that the recruitment of these patients bridged the period during which mutations in *CALR* were identified in ET and MF patients (Klampfl *et al.*, 2013, Nangalia *et al.*, 2013). Although all *JAK2*/*MPL* negative patients were retested for *CALR*, it is possible that mutants may be missed in some of these patients due to limits of detection in current diagnostic methods in detecting low-allele burden samples (Lim *et al.*, 2015, Luo and Yu, 2015). Recently, whole exome sequencing of triple-negative samples from ET and MF patients identified a small number of patients with activating mutations outside of exon 10 of *MPL* and novel *JAK2* mutations that could constitutively activate JAK2-STAT5 signalling (Milosevic Feenstra *et al.*, 2016). This indicates that caution should be taken when drawing conclusions from comparisons between *JAK2/CALR/MPL* mutated, and triple-negative patients, as discrete homogenous groups.

The results showed no significant difference in platelet counts between the mutation types, whereas previous groups have shown that platelet counts are significantly higher in *CALR* vs both *JAK2*^{V617F} and triple-negative patients (Rotunno *et al.*, 2014, Rumi *et al.*, 2014a). However, it was only the *JAK2*^{V617F}

mutation that was associated with a higher risk of thrombotic events (Rotunno *et al.*, 2014). It should be noted that the results here are for patients undergoing a range of cytoreductive and platelet lowering therapies, which, depending on their efficacy, may have a confounding effect on the results. Also, as previously mentioned, the triple-negative cohort of patients is likely to be a heterogenous group whose activating mutations may share similarities with either *JAK2*^{V617F}*, CALR* exon 9*,* or *MPL*$^{W515K/L}$. It is also very likely that no significant differences were observed due to the small sample size. Previous studies demonstrating lower platelet counts in *CALR* mutated ET patients have used data from over 600 patients (Rumi *et al.*, 2014a).

This study has shown that *GATA1* is overexpressed in the peripheral blood of ET patients and this occurs independently of mutational status. *GATA1* has previously been shown to be significantly upregulated in the bone marrow of ET and PV patients, but not in MF (Rinaldi *et al.*, 2008) and the results in peripheral blood of ET patients reflect these findings. The increase in *GATA1* is independent of platelet-lowering and cytoreductive therapy, which may suggest a role as a marker for disease. Interestingly, a moderate correlation was found between increased platelet counts and lower (although still upregulated) *GATA1* expression. Studies separating the ET category of disease into two distinct groups, “true-ET” and “prefibrotic-MF (pre-MF)”, have shown that the pre-MF group have similar (Barosi *et al.*, 2012) or even significantly increased (Barbui *et al.*, 2011, Rupoli *et al.*, 2015) platelet counts versus true-ET. It is possible that reduced *GATA1* expression (relative to other ET patients) may be indicative of a pre-MF phenotype. Further loss of *GATA1* expression and relative downregulation may also point towards disease transformation to myelofibrosis, where impaired haematopoiesis is driven by low *GATA1* levels (Gilles *et al.*, 2017). This cohort of patients were recruited according to the 2008 WHO criteria for ET, it may be useful to retrospectively analyse this group and identify whether any fall into the recently designated pre-MF category (Arber *et al.*, 2016). Samples with much higher platelet counts or existing patients whose platelet counts have increased, may be required to confirm whether there is any correlation with *GATA1* gene expression.

NFE2, a key regulator of megakaryopoiesis, did not show any change in level of expression. This is surprising, since firstly the p45 subunit of NFE2 is a direct transcriptional target of GATA1 (Tsang *et al.*, 1997), and secondly, previous studies have shown that *NFE2* is overexpressed in both PV and ET patients (Wang *et al.*, 2010). These results were from mRNA extracted from granulocytes (Wang *et al.*, 2010) rather than PBMCs which were used in this study. The differences in cell populations between the two methods and presence of earlier progenitors in PBMCs may explain why our results did not reflect these findings. NFE2 is associated with late-stage megakaryocytic development and *NFE2* knockout is associated with defects in proplatelet formation and platelet shedding (Tijssen and Ghevaert, 2013). *NFE2* did show a moderate positive correlation with platelet levels, which suggests that regulation of *NFE2* affects disease pathogenesis. From our results, we conclude that expression of *NFE2* and its effect on platelet levels is independent from *GATA1* expression in these cells.

FLI1 was shown to be significantly downregulated in peripheral blood mononuclear cells derived from ET patients. This is unusual since other research has demonstrated that it is reduced in the bone marrow of ET patients (Bock *et al.*, 2006). Cross-antagonism between the erythroid specific transcription factor, EKLF, and FLI1 has also been shown to promote megakaryopoiesis (Siatecka *et al.*, 2007, Starck *et al.*, 2003). Furthermore, FLI1 is required for transcriptional synergy between GATA1 and FOG1 in upregulating expression of megakaryocytic specific genes (Wang *et al.*, 2002). Given that the primary hallmark of ET is increased platelet count and megakaryocyte proliferation, the decrease in FLI1 expression in these patients does not appear to negatively impact this. *FLI1*, unlike both *GATA1* and *NFE2*, did not correlate with measured platelet counts or with the expression of either *GATA1* or *NFE2.*

The functions of calreticulin, in calcium homeostasis and as an ER chaperone protein, are understood (Michalak *et al.*, 2009) but it is not yet fully known how mutations in *CALR* that are found in MPN patients result in an ET or MF (but not PV) disease phenotype. Recently, the type of calreticulin mutation (52bp deletion or 5bp insertion) has been shown to have differential effects on the

phenotype (ET versus MF) as well as the risk of myelofibrotic transformation (Cabagnols *et al.*, 2015). Ruxolitinib (a JAK 1/2 inhibitor) is equally effective in treating *JAK2* mutated and non-mutated MPN, indicating that all these mutations (*JAK2*^{V617F}, exon 12, *MPL* and *CALR*) affect a common pathogenic pathway. The reduction in gene expression in *CALR*, in all mutation types, suggests that loss of *CALR* function may be responsible in part for the MPN phenotype (in contrast to the gain of function seen in *JAK2* and *MPL* mutations). Other groups have reported that *CALR* is lower in MPN patients compared to healthy volunteer donors (Park *et al.*, 2014). Interestingly this group also reported that *CALR* expression was higher (not significant) in non-*JAK2* mutated than in *JAK2*^{V617F} or exon 12 mutated patients (Park *et al.*, 2014). This is contrary to our results which showed that *JAK2* mutations were associated with increased *CALR* levels over triple-negative patients (significant). However, the differences in methodology between the two studies should be noted, our study examined ET patients only whereas the data by Park *et al.* (2014) included PV and MPN-unclassified as well as ET patients. It was also not specified in the study whether the RNA was extracted from bone marrow aspirates or peripheral blood. Park *et al.* (2014) also highlight that their results were not statistically significant, probably due to the low number of MPN patients studied.

These results demonstrate that *GATA1* is a useful marker of ET disease, especially since it is still overexpressed while patients are undergoing cytoreductive or platelet lowering therapies. Further investigation may be required to determine whether newer JAK2 kinase inhibitors, such as ruxolitinib, have any effect on *GATA1* expression levels. It has also been shown that increased *GATA1* mRNA does not directly alter the expression of related haematopoietic genes, *NFE2* and *FLI1*. Finally, calreticulin is significantly downregulated, along with another chaperone protein, calnexin, suggesting that post translational modifications and quality control in the endoplasmic reticulum may play a role in ET disease, independent of mutational status.

CHAPTER 4: Molecular mechanisms of *GATA1* in MPN cell models

4.1: Introduction

To explore the role of GATA1 in ET patients, it was decided to study its mechanisms in cell line models treated with anagrelide. Unlike hydroxyurea, the gold standard therapy used in ET (Harrison and Keohane, 2013), anagrelide is not a cytoreductive agent and works by inhibiting megakaryopoiesis (Ahluwalia *et al.*, 2010), although the exact mechanism of action is not fully understood. Anagrelide is a potent phosphodiesterase III (PDE3) inhibitor, which can prevent aggregation of platelets – although this is only observed in doses that are higher than clinically relevant (Balduini *et al.*, 1992). Studies have demonstrated a possible mechanism of action involving a down-regulation of *GATA1* expression (Ahluwalia *et al.*, 2010). Anagrelide does not affect MPL signal transduction, JAK2 and STAT3 phosphorylation levels are unchanged in cell lines exposed to the drug, but during TPO-induced megakaryopoiesis, expression levels of *GATA1* and *FOG1* are reduced (Ahluwalia *et al.*, 2010).

Five cell line models were used in these experiments, three of these (HEL, SET2 and UKE1) contain the $JAK2^{V617F}$ mutation found in 50-60% of cases of ET. Two of the cell lines (SET2 and UKE1) were derived from patients with a prior history of ET before transformation to AML (Uozumi *et al.*, 2000, Fiedler *et al.*, 2000). UKE1 and HEL were homozygous for the $JAK2^{V617F}$ mutation whilst SET2 expressed both $JAK2^{V617F}$ and $JAK2^{WT}$ (Quentmeier *et al.*, 2006).

The roles of GATA1 and FOG1 in haematopoiesis have been discussed previously in Chapter 3. *FOG1* is co-expressed with *GATA1* during haematopoiesis, and through its binding on one of the N-terminal fingers on GATA1, is responsible for normal erythroid and megakaryocyte development (Tsang *et al.*, 1998, Tsang *et al.*, 1997). Both *FOG1* knockout mouse models and mutants affecting GATA1/2 binding to FOG result in megakaryopoietic failure. FOG1 may also have an independent role from GATA1 in inhibiting differentiation in mast cell and eosinophil lineages (Chlon and Crispino, 2012), exogenous expression of *FOG1* in these eosinophil progenitors results in erythro-megakaryocyte features (Querfurth *et al.*, 2000).

GATA2 expression overlaps with that of *GATA1* and during terminal erythroid differentiation there is a switch from *GATA2* to *GATA1* expression (Cheng *et al.*, 1996, Suzuki *et al.*, 2013). Cell lines with high levels of GATA2 show a decrease in proliferation and a shift towards a megakaryocyte lineage, including an increase in ploidy (Ikonomi *et al.*, 2000). *GATA2* overexpression along with *GATA1* mutations are associated with paediatric Down's syndrome acute megakaryoblastic leukaemia (DS-AMKL) (Huang and Crispino, 2015). It also controls cell cycle progression in GATA1 deficient cells, while overexpression enhances megakaryocyte development (Huang *et al.*, 2009).

NFE2 is a heterodimeric transcription factor containing haematopoietic specific p45 subunit and a smaller Maf protein (Andrews *et al.*, 1993). *p45* knockout mice do not develop platelets and die from haemorrhage, although there is still megakaryocyte proliferation in response to TPO (Shivdasani *et al.*, 1995). The p45 subunit of NFE2 is under direct transcriptional control of GATA1, platelets deficient in GATA1 have lower expression levels of *p45 NFE2* (Vyas *et al.*, 1999).

PU.1 is a haematopoietic transcription factor involved in terminal myeloid cell maturation, B-cell, and T-cell development (Kastner and Chan, 2008). It is also expressed in early erythroid progenitor cells and is downregulated during terminal differentiation (Back *et al.*, 2004). GATA1 directly interacts with the PU.1 protein and the two antagonise each other's functions (Nerlov *et al.*, 2000, Zhang *et al.*, 2000).

Platelet factor 4 (PF4) is a CXC family chemokine expressed by megakaryocytes and released during platelet aggregation (Xia and Kao, 2003). It has a high affinity for, and forms an inactivating complex with, heparin (Denton *et al.*, 1983). GATA1 has been shown to interact with the promoter region of *PF4* (Minami *et al.*, 1998). Disruption of the GATA binding motif on the *PF4* promoter reduces transcriptional activity of a reporter gene while overexpression of *GATA1* enhances it (Minami *et al.*, 1998).

PSTPIP2 is a target gene for GATA1 and is upregulated in *GATA1*-mutated or low *GATA1* expressing megakaryocytes (Liu *et al.*, 2014). During TPA-induced megakaryopoiesis in K562 cells, *PSTPIP2* is upregulated with a concomitant fall in the expression of *GATA1* (Liu *et al.*, 2012).

The glycoprotein IX (GPIX) protein is found on the surface of platelets (Li and Emsley, 2013). Its expression is associated with late stage megakaryopoiesis (Tijssen and Ghevaert, 2013). FLI1 in synergy with GATA1 binds to promoter sequences upstream from the *GPIX* gene and enhances expression (Eisbacher *et al.*, 2003).

4.2: Aims

Following on from the experiments investigating *GATA1* expression in ET patients, it was decided to examine *GATA1*, associated transcription factors and markers of differentiation involved in megakaryopoiesis in cell models. Anagrelide was used to probe *GATA1* in cell line models, as it has previously been reported to affect the expression of *GATA1* and other haematopoietic genes (Ahluwalia *et al.*, 2010). These experiments in cell lines, including AML transformed ET models, will provide further understanding how dysregulated GATA1 signalling can contribute to disease pathogenesis in ET. *GATA1* regulation by anagrelide will also be studied to determine whether it is directly responsible for its anti-platelet activity *in-vivo*.

4.3: Methods

Methods are described in brief here, for full methodology refer to Chapter 2.

MTS Assays: Cells (K562, HL-60, HEL, SET2 and UKE1) were incubated for 72 hours in the presence of inhibitors and vehicle control (DMSO). After incubation, 20 µL of MTS (Promega, Madison, USA) with PMS (Sigma-Aldrich, Poole, UK) (50:1) solution was added to 100 µL of cells in culture media. Cells were incubated for 2 – 4 hours and absorbance at 490 nm was measured. Control wells were treated with 10% Triton-X100 (Sigma) 20 minutes prior to addition of PMS for complete cell death.

Trypan blue exclusion assay: Cells were cultured in the presence of anagrelide for 96 hours. Samples were taken at each 24-hour timepoint and mixed with 0.4% trypan blue (Sigma-Aldrich, Poole, UK). Cell counts (live and dead) were taken with an inverted microscope and haemocytometer.

Cell cycle analysis: Cell lines (K562 and HEL) were serum-starved overnight prior to treatment with anagrelide. After 48 hours of treatment, cells were collected by centrifugation and washed with ice-cold PBS. Cell fixation and propidium iodide staining were performed as per published protocols (Darzynkiewicz and Juan, 2001). Cell fluorescence was measured on a FACSVerse (BD Biosciences) and analysis carried out using FlowJo VX software (Treestar Inc).

Transcriptome analysis (*in-vitro* cell line models): Cell lines (K562 and HEL) were treated with anagrelide (± 1 μM). After 48 hours, RNA was isolated (Qiagen, Hilden, Germany) and cDNA was synthesised (Bio-Rad, Hercules, USA). Relative quantification of key haematopoietic genes was determined on a StepOne Plus real time PCR instrument (Applied Biosystems) using Sybr Green I mastermix (Bio-Rad, Hercules, USA).

Phorbol 12-myristate 13-acetate (PMA) induced cell differentiation: K562 and HEL cells were treated with 1×10^{-8} M PMA to induce megakaryopoietic differentiation. After 48 hours, cell morphology was examined under an inverted microscope. Cells were gently dislodged from the culture plate surface by pipetting and light scraping. Collected cells were spun down and transcriptome analysis carried out as described previously.

4.4: Results

4.4.1: Cellular proliferation in the HEL cell line is significantly reduced by anagrelide treatment

Five cell lines (SET2, UKE1, HEL, K562, and HL-60) were exposed to increasing concentrations of the anti-platelet drug, anagrelide, over 72 hours. The erythroleukaemic cell line, HEL, showed significant reduction in proliferation at 0.01 µM ($p < 0.05$) and a 50% reduction compared to DMSO control at 100 µM (Figure 25). HEL was the only *JAK2*^{V617F} cell line to respond to anagrelide treatment. Neither SET2 (Figure 26) nor UKE1 (Figure 27) showed any significant change ($p > 0.05$) in proliferation to anagrelide. Therefore, IC_{50} values for this drug were unable to be calculated for both these cell lines.

Both *JAK2*^{WT} cell lines, K562 (Figure 28) and HL60 (Figure 29), did not respond to treatment. No significant difference (K562: $p > 0.05$; HEL: $p > 0.05$) in proliferation was observed at the highest concentration tested. As previous with SET2 and UKE1, IC_{50} values were not determined.

The presence of a *JAK2* mutation, also seen in SET2 and UKE1, does not appear to be responsible for the sensitivity of the HEL cell line to anagrelide. Also, loss of heterogeneity (LOH) of JAK2 in the HEL cell line is unlikely to have any effect on sensitivity to anagrelide since UKE1 is also homozygous for the *JAK2*^{V617F} mutation.

Anagrelide does not appear to have any anti-proliferative activity in the SET2, UKE1, K562, or HL60 cell lines and its effects on HEL are likely to be unique to this cell line and unrelated to aberrant JAK2 signalling.

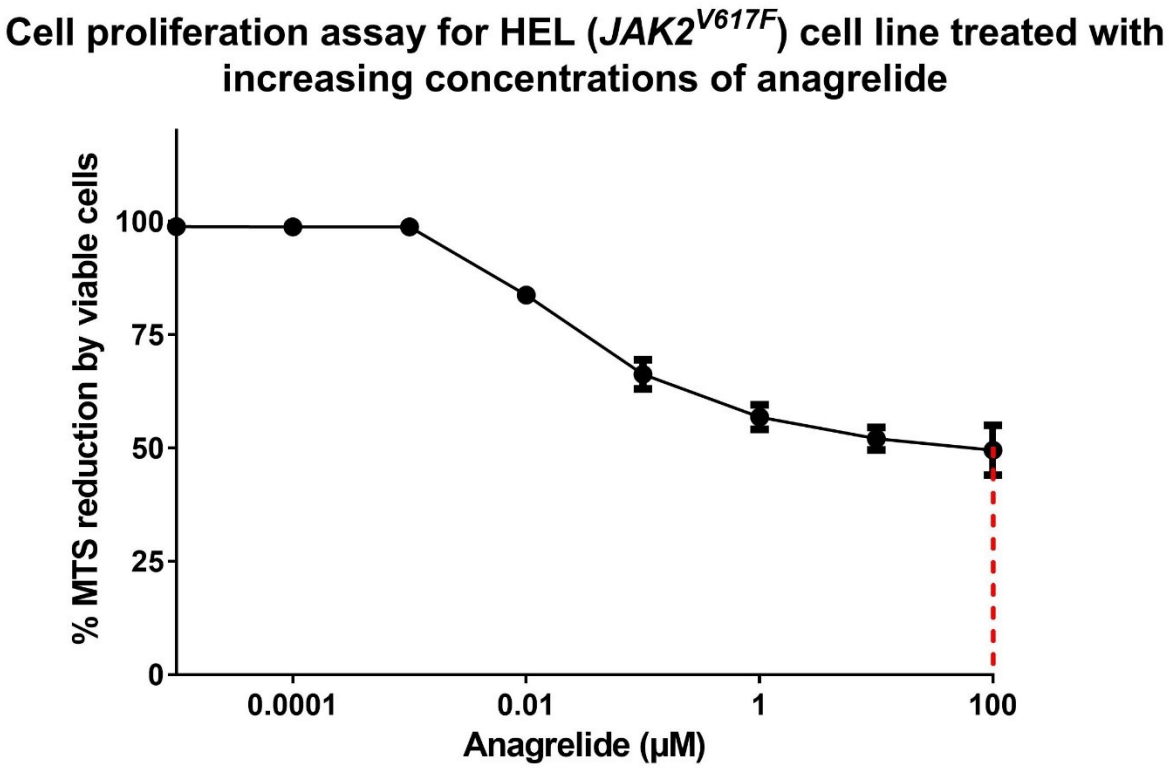


Figure 25: Cellular proliferation assay for anagrelide in the HEL cell line with *JAK2*^{V617F} mutation. IC_{50} = 100 µM. Significance (α = 0.05) between highest and lowest concentrations calculated using t-test ($p < 0.05$). Experiment carried out at three independent times (n = 3 ± SD).

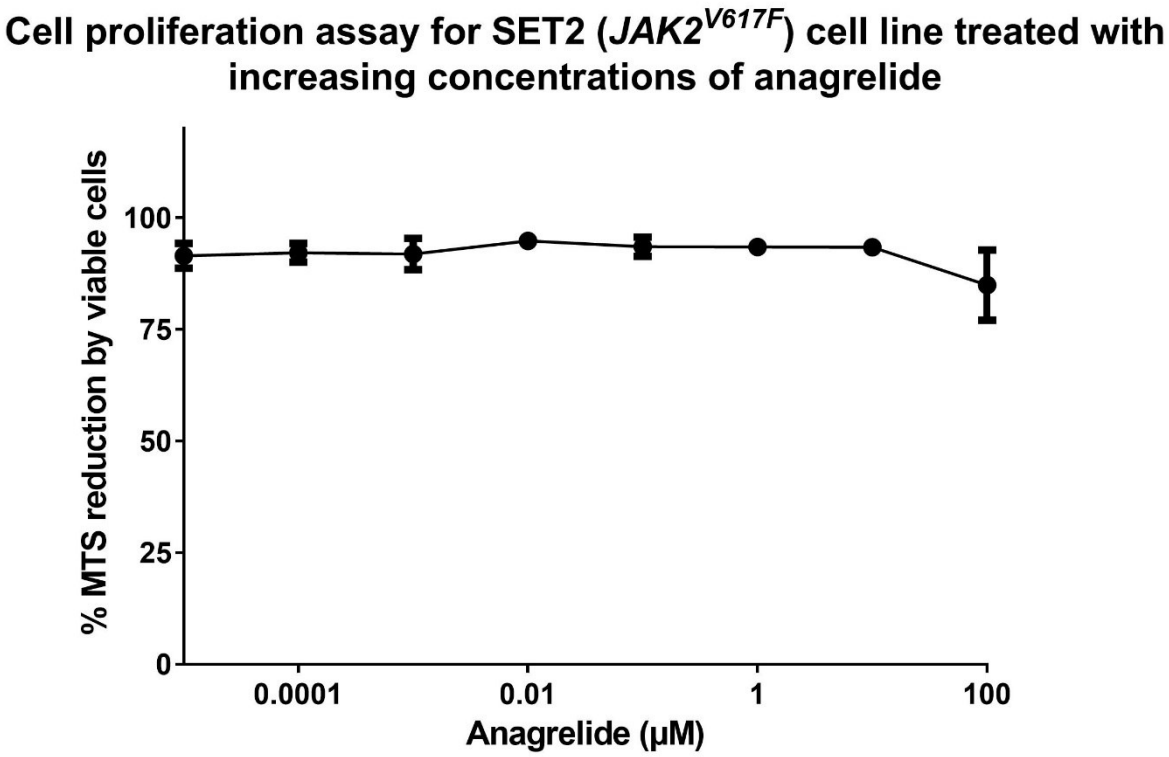


Figure 26: Cellular proliferation assay for anagrelide in the SET2 cell line with *JAK2*^{V617F} mutation. Cell viability, measured by MTS reduction, was not affected by anagrelide treatment. No significant difference ($\alpha = 0.05$) at highest concentration (t-test; $p > 0.05$). Experiment carried out at three independent times (n = 3 ± SD).

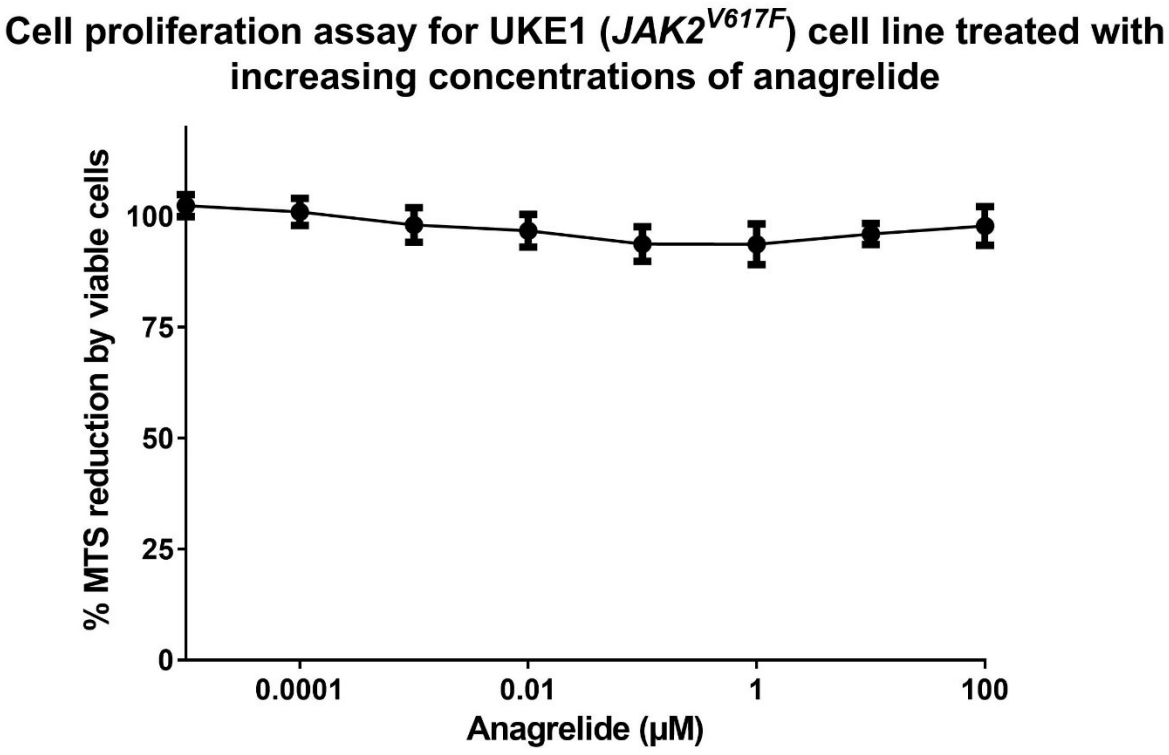


Figure 27: Cellular proliferation assay for anagrelide in the UKE1 cell line with *JAK2*^{V617F} mutation. Cell viability, measured by MTS reduction, was not affected by anagrelide treatment. No significant difference ($\alpha = 0.05$) at highest concentration (t-test; $p > 0.05$). Experiment carried out at three independent times (n = 3 ± SD).

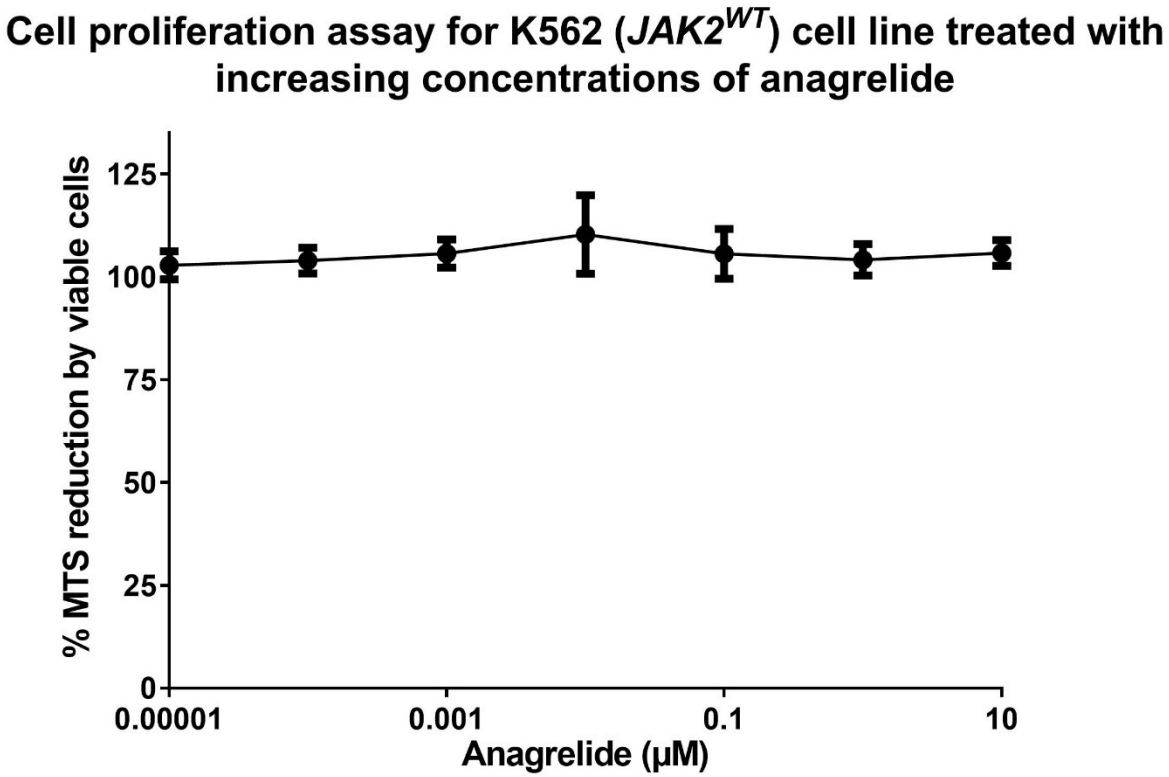


Figure 28: Cellular proliferation assay for anagrelide in the JAK^{WT} cell line, K562. Cell viability, measured by MTS reduction, was not affected by anagrelide treatment. No significant difference (α = 0.05) at highest concentration (t-test; $p > 0.05$). Experiment carried out in triplicate (n = 3 ± SD).

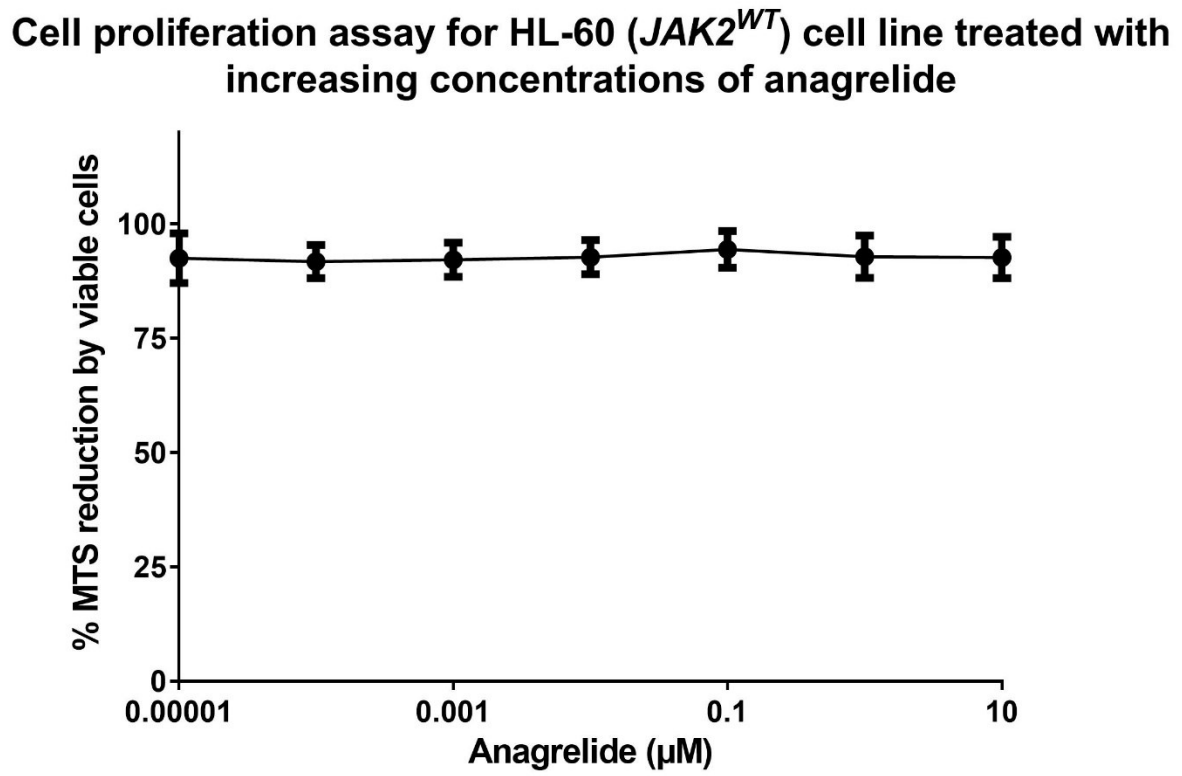


Figure 29: Cellular proliferation assay for anagrelide in the *JAK*^{WT} cell line, HL60. Cell viability, measured by MTS reduction, was not affected by anagrelide treatment. No significant difference (α = 0.05) at highest concentration (t-test; $p > 0.05$). Experiment carried out in triplicate (n = 3 ± SD).

4.4.2: Anagrelide treatment results in an increase in cells in the G0/G1 phase of the cell cycle

Following on from the cell proliferation experiments, two cell lines (K562 and HEL) were selected for cell cycle analysis, using propidium iodide staining. After treatment for 48 hours with 1 µM anagrelide, followed by fixation in ethanol, no significant change was observed between fractions of K562 cells in each phase of the cell cycle (Figure 30 and Figure 31). T-test results: G0/G1: $p = 0.1539$; S: $p = 0.6032$; G2/M: $p = 0.1464$ (Figure 30).

HEL cells, which responded to anagrelide in cell proliferation assays (Figure 24), showed a significant increase (Figure 32 and Figure 33) in the proportion of cells in G0/G1 phase (t-test; $p = 0.0224$) (Figure 32). This was followed by decreases in the proportion of cells in both S ($p = 0.0096$) and G2/M phases ($p = 0.0037$) (Figure 33). Further work using the trypan blue exclusion assay resulted in a reduction in cell growth over 96 hours (> 4-fold) and a significant decrease in cell viability for cells treated with anagrelide compared to control (Figure 34). At 72 hours, cells treated with 0.3 µM anagrelide had cell viability of 56% versus 92% in the DMSO vehicle control group (Figure 34).

The anti-proliferative effect of anagrelide appears to be specific to the HEL cell line and involves a block in cell cycle progression from G1 to S phase. This also results in cytotoxicity, only observed in the HEL cell line. It is not yet known whether this mechanism plays any role in the anti-megakaryopoietic activity the drug has in MPN patients.

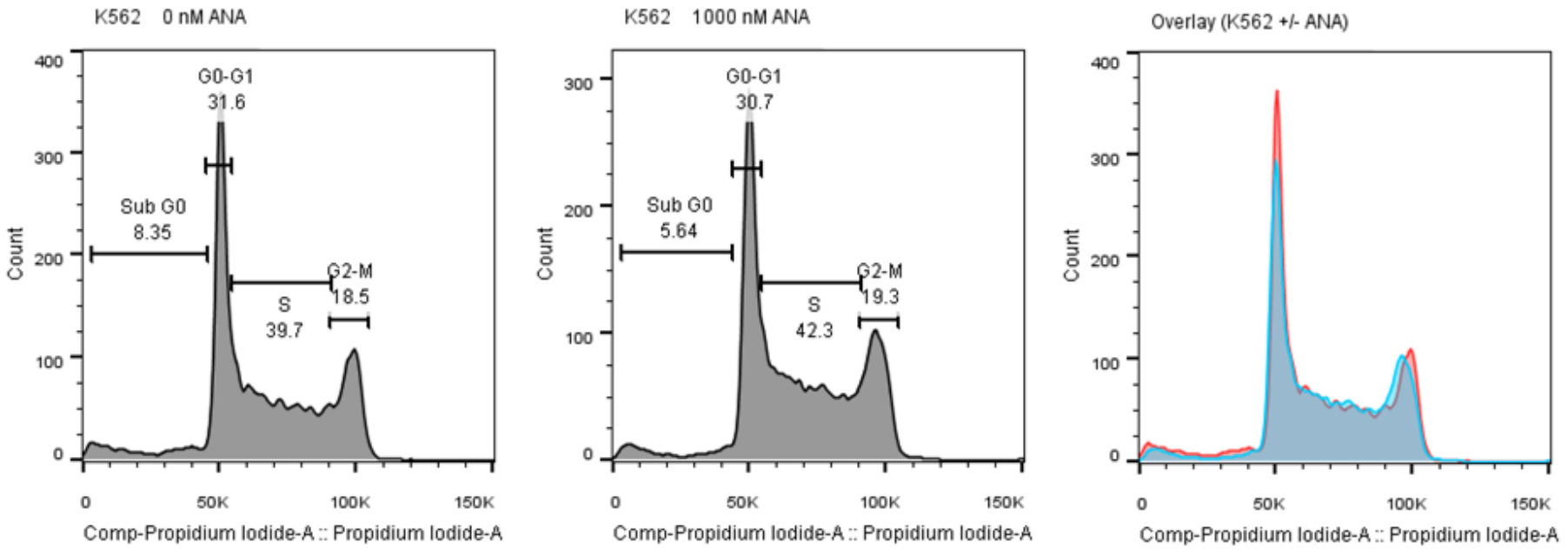


Figure 30: Effect of anagrelide on cell cycle in K562 cells. Representative experiment shown in the figure (FlowJo Software), experiment was carried out in triplicate. No difference was observed in the proportion of K562 cells in each stage of the cell cycle ± anagrelide.

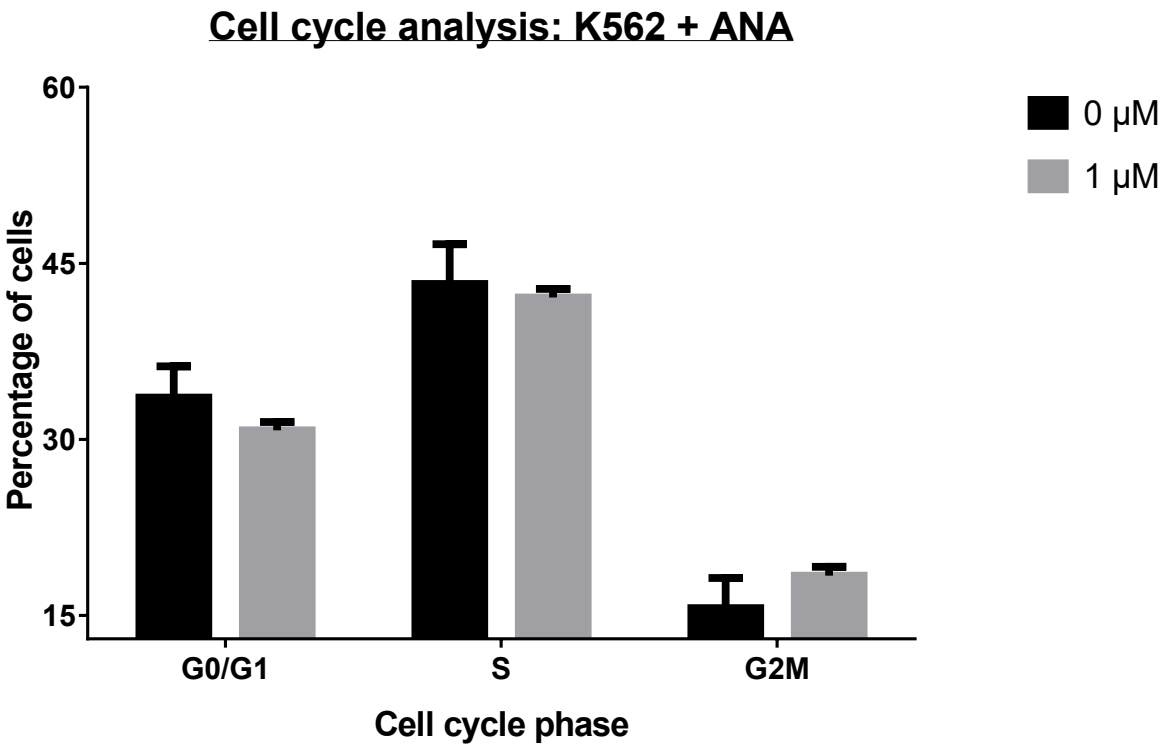


Figure 31: Cell cycle analysis of K562 cells treated with 1 µM anagrelide for 48 hours. Bars show average percentage cells in each phase of the cell cycle with standard deviation (n = 3 ± SD). Anagrelide did not have any significant effect (t-test; $p > 0.05$) on cell cycle phases in the K562 cell line.

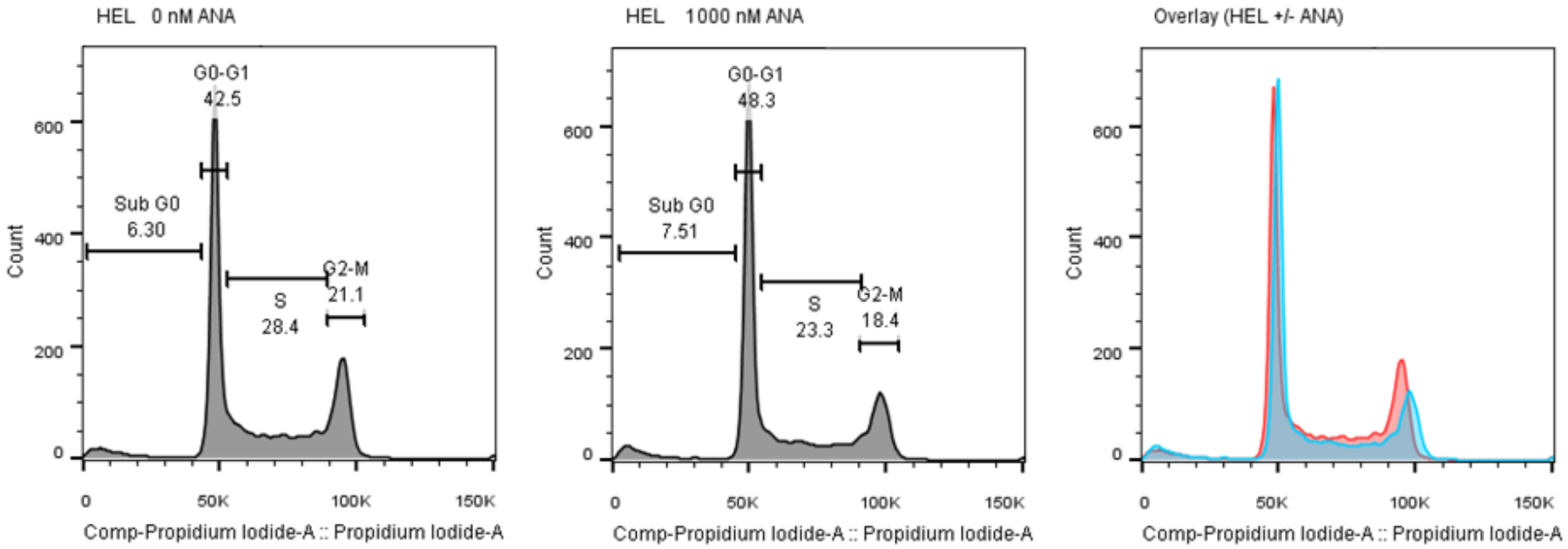


Figure 32: Effect of anagrelide on cell cycle in HEL cells. Representative experiment shown in the figure (FlowJo Software), experiment was carried out in triplicate. There was a decrease in cells in S and G2M phase for HEL cells treated with anagrelide compared to control.

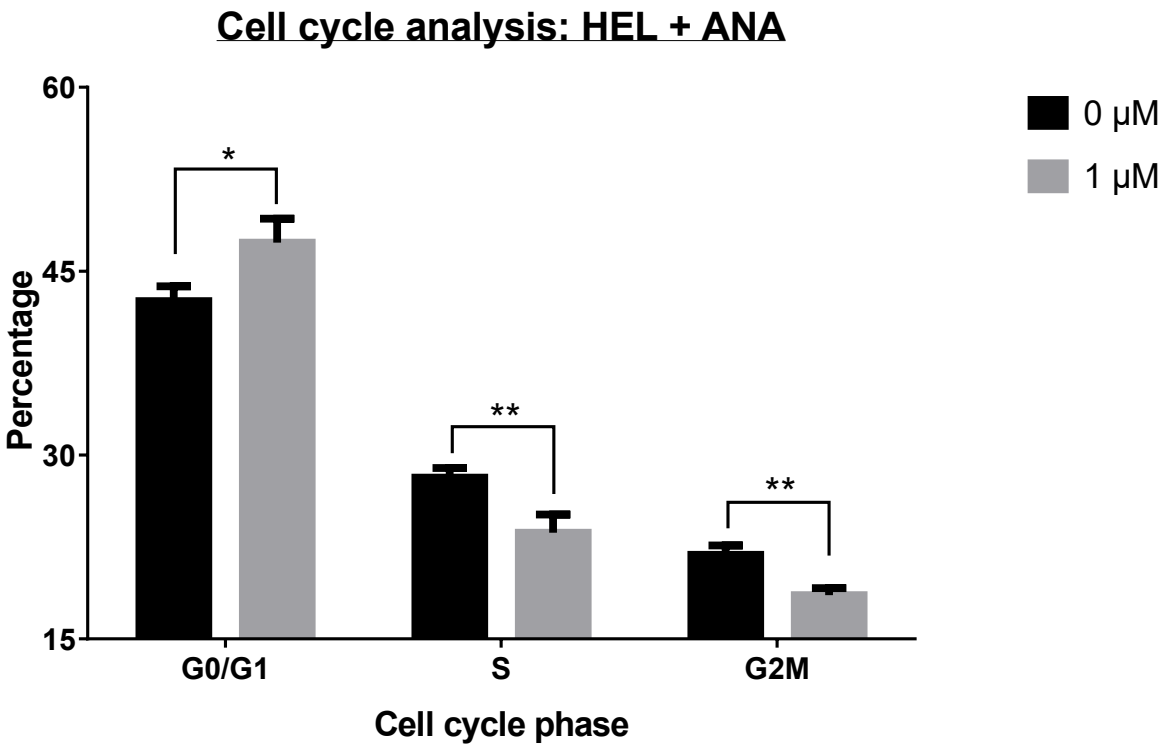


Figure 33: Cell cycle analysis of HEL cells treated with 1 µM anagrelide for 48 hours. Bars show average percentage cells in each phase of the cell cycle with standard deviation (n =3 ± SD). In HEL treated cells, a significant increase (α = 0.05) was observed in number of cells in G0/G1 phase (t-test; $p < 0.05$) on anagrelide treatment. Conversely significant decreases were measured in cell numbers entering S (t-test; $p < 0.05$) and G2/M (t-test; $p < 0.05$) phases.

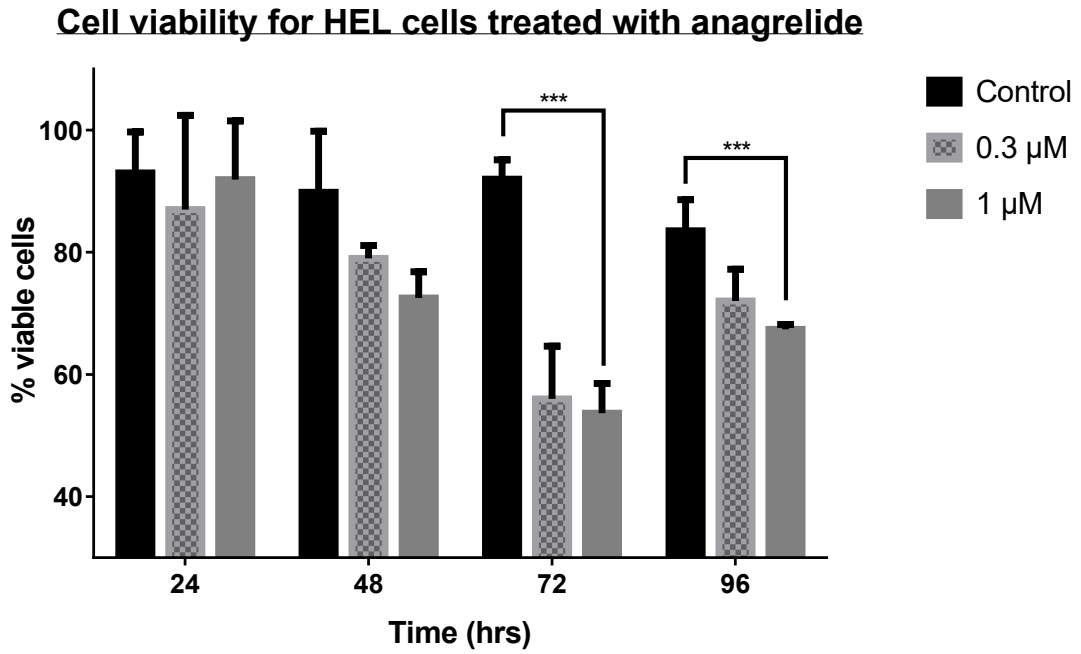


Figure 34: Cell viability calculated using trypan blue exclusion assay. Total (live and dead) cell counts taken at each 24-hour time point (n = 3 ± SD). Percentage of live cells per total cell number determined. There was a significant (α = 0.05) decrease in viability for cells treated with 1 µM anagrelide after 72 hours (t-test; $p < 0.05$).

4.4.3: Anagrelide has no effect on the expression of key haematopoietic genes in cell models

Key haematopoietic gene expression and response to anagrelide was examined in three cell lines, two *JAK2*^{V617F} cell lines, HEL and SET2, and a control *JAK2*^{WT} cell line, K562.

HEL previously showed proliferation and cell cycle responses when exposed to 1 µM of anagrelide (Figure 24, Figure 31 and Figure 32). Real time PCR studies with this cell line failed to show any significant changes (t-test; $p > 0.05$) to gene expression in any of the following genes, *GATA1, GATA2, FOG1, NFE2* and *PU.1* (Figure 35). The other two cell lines tested, SET2 (Figure 36) and K562 (Figure 37), also did not show any significant up or down regulation of these genes ($p > 0.05$).

These results suggest that the factors that inhibit proliferation and induce cell death in HEL are not caused by direct *GATA1* or haematopoietic gene regulation by anagrelide. It may also indicate that any activity of anagrelide on *GATA1* may be restricted to certain differentiation specific events.

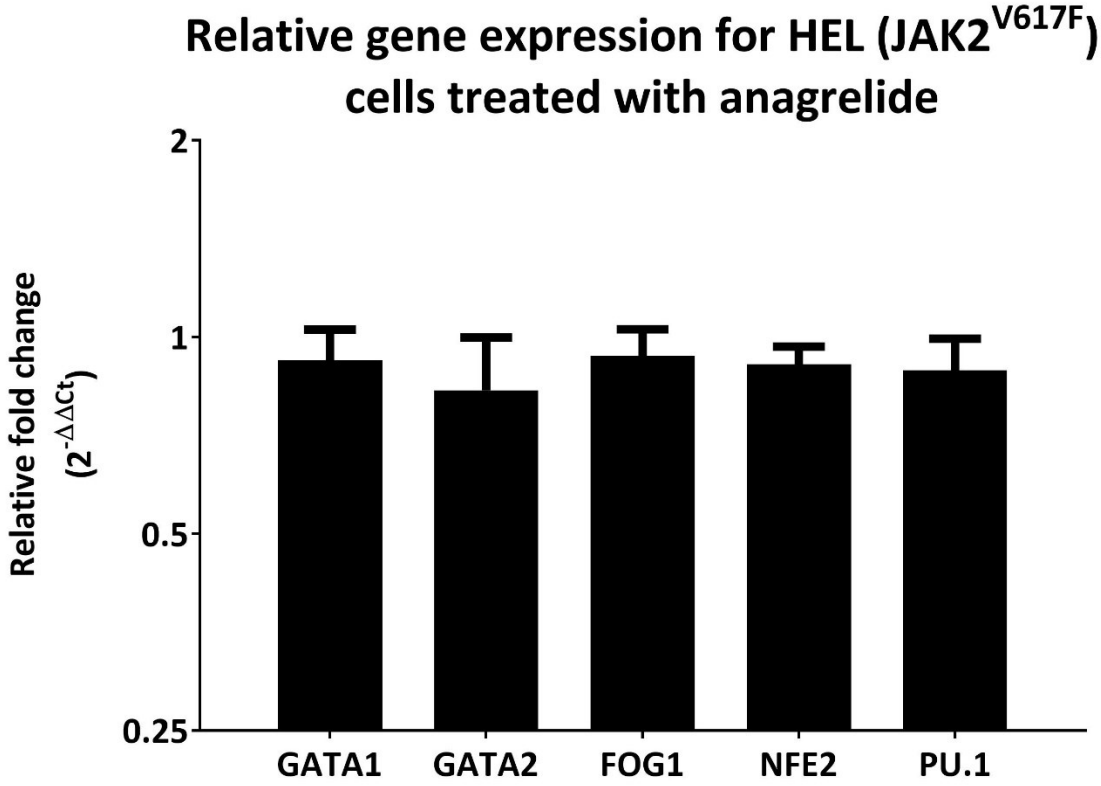


Figure 35: Relative fold change of key haematopoietic genes in the homozygous *JAK2*^{V617F} cell line, HEL, treated with 1 µM anagrelide (72 hours) (n = 3 ± SD). Expression is relative to the housekeeping gene, *GAPDH*, and a vehicle control sample (DMSO). No significant change in expression observed for any gene tested (t-test; $p > 0.05$).

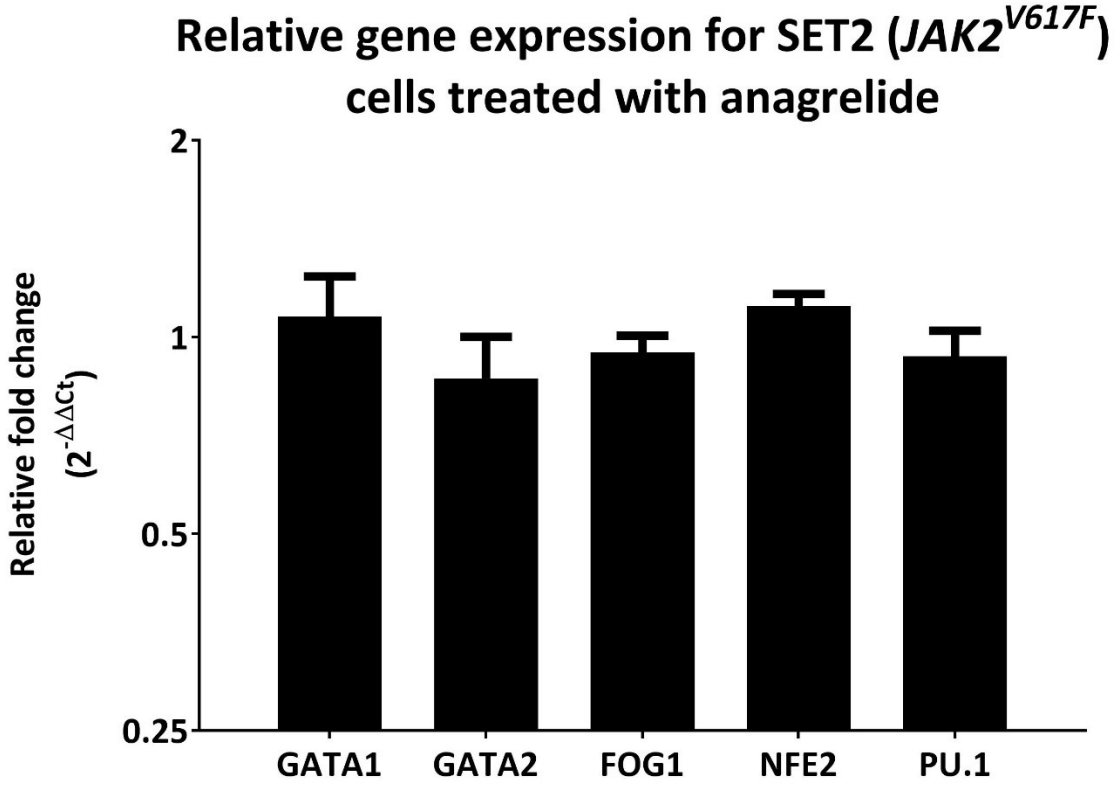


Figure 36: Relative fold change of key haematopoietic genes in the heterozygous *JAK2*^{V617F} cell line, SET2, treated with 1 µM anagrelide (72 hours) (n = 3 ± SD). Expression is relative to the housekeeping gene, *GAPDH*, and a vehicle control sample (DMSO). No significant change in expression observed for any gene tested (t-test; $p > 0.05$).

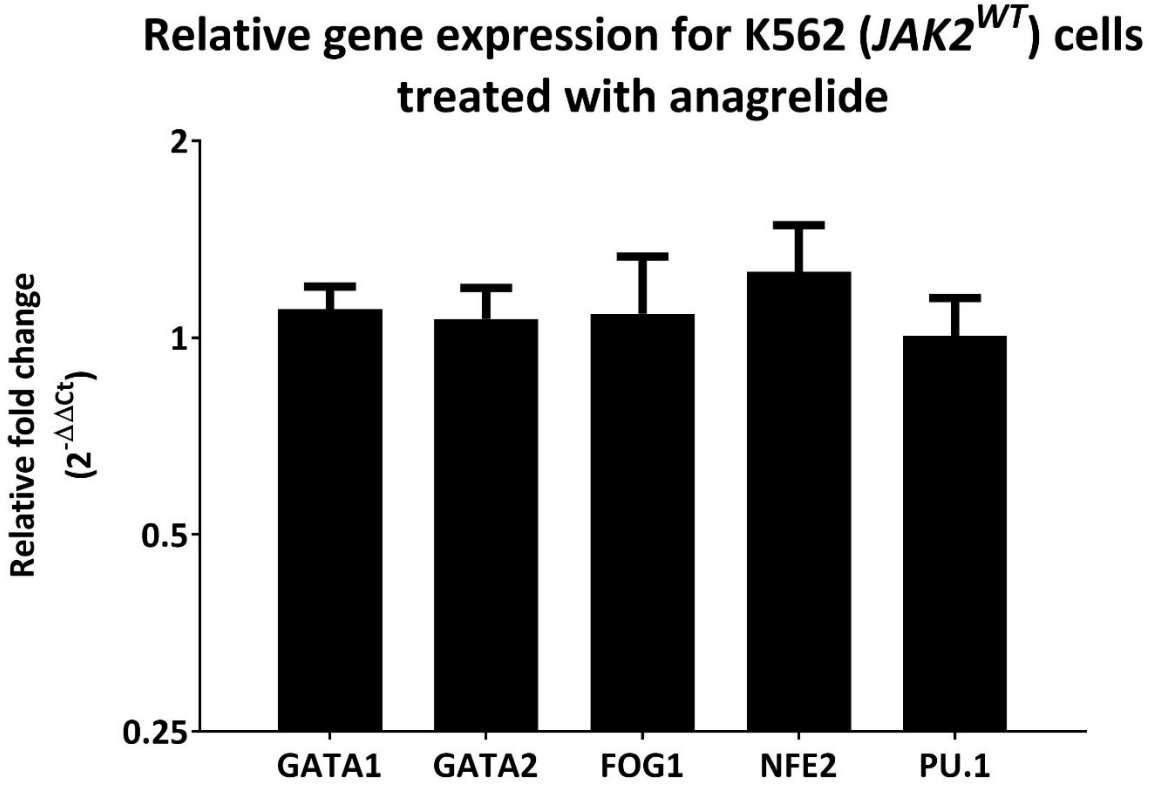


Figure 37: Relative fold change of key haematopoietic genes in the *JAK*^{WT} cell line, K562, treated with 1 µM anagrelide (72 hours) (n = 3 ± SD). Expression is relative to the housekeeping gene, *GAPDH*, and a vehicle control sample (DMSO). No significant change in expression observed for any gene tested (t-test; $p > 0.05$).

4.4.4: Anagrelide has no effect on Phorbol 12-myristate 13-acetate (PMA) induced differentiation

To investigate whether anagrelide may have an effect during induced differentiation, the suspension cell lines K562 and HEL were treated with 1 x 10^{-8} M PMA ± 1 µM anagrelide. PMA arrested cell growth and resulted in changes to cell morphology and adhesion to the culture plate surface. These were observed under 10X magnification on an inverted microscope. After 48 hours, no difference in cell adhesion to the plate surface was observed between PMA and PMA + ANA in either cell line (Figure 38). The anti-proliferative effect of anagrelide on HEL cells, as demonstrated in Figure 25 and Figure 34 was not observed whilst cells were induced to differentiate. Anagrelide, at the concentration tested, does not appear to reduce megakaryocytic differentiation induced by PMA.

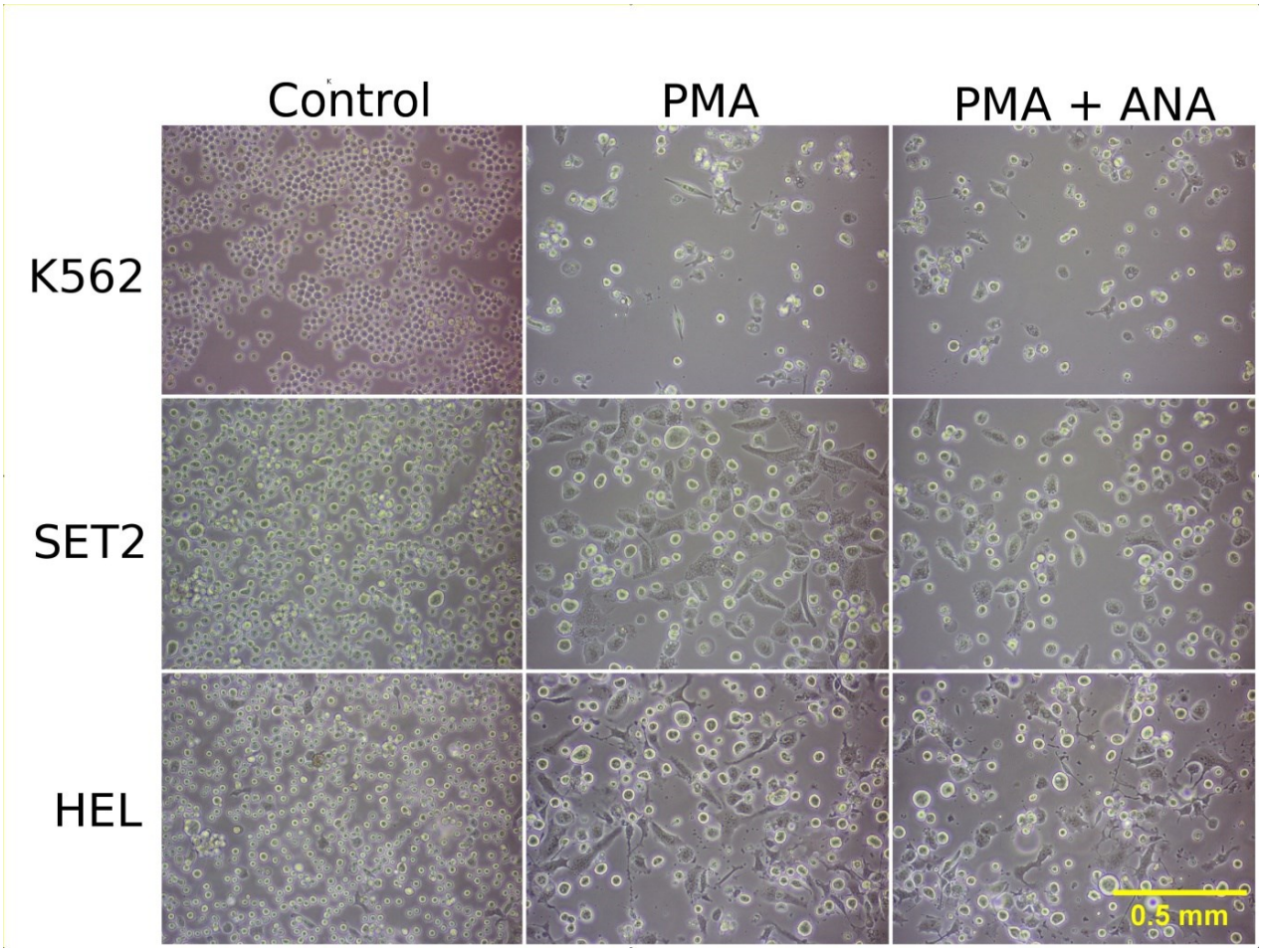


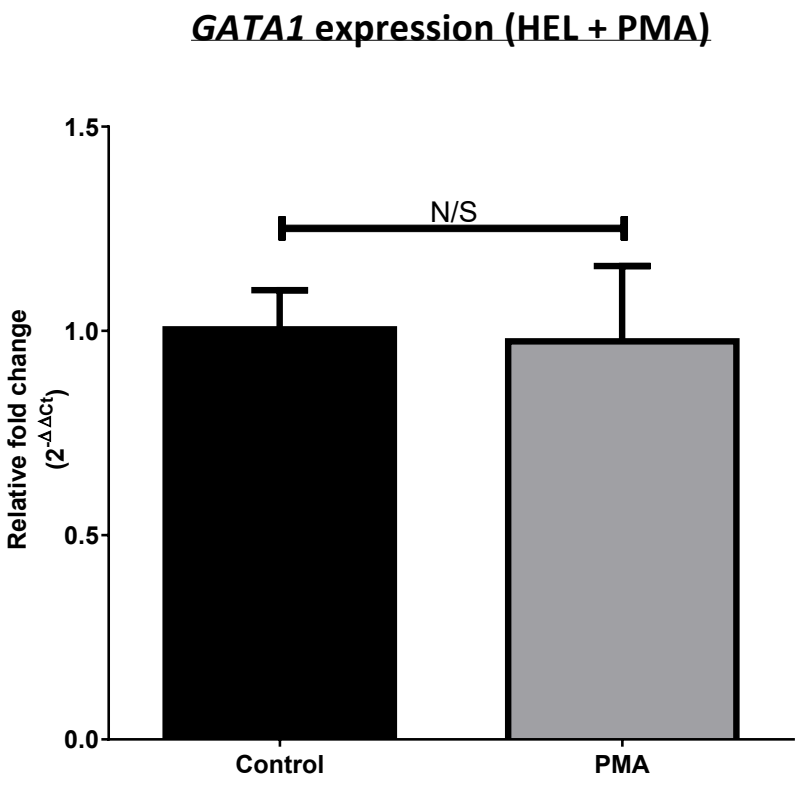
Figure 38: Effect of anagrelide on morphology changes induced by PMA treatment. Cells (K562, HEL and SET2) induced to differentiate in the presence of PMA (1 x 10^{-8}M) ± anagrelide (1 µM). Differentiation is noted by change in morphology and adherence to surface of culture dish. DMSO used as vehicle control for PMA and anagrelide.

4.4.5: Anagrelide reduced gene expression of the megakaryocyte markers PF4 and PSTPIP2 during PMA induced differentiation

GPIX, PF4 and *PSTPIP2* were selected as markers for differentiation induced by PMA treatment. Expression of *PSTPIP2* and *GPIX* are under direct transcriptional control by *GATA1* with *GPIX* modulated by the interaction of *GATA1* and its co-factor, *FOG1*.

No change ($p > 0.05$) was observed in *GATA1* expression between control (undifferentiated) cells and those exposed to PMA (Figure 39). Concomitant treatment with anagrelide and PMA compared to vehicle control (DMSO) and PMA also showed no significant change ($p > 0.05$) in *GATA1* levels (Figure 40). PMA treatment resulted in a 2.5-fold increase in the relative level of *GPIX* ($p < 0.05$) during induced differentiation (Figure 41), although on exposure to anagrelide over the 48 hours there was no significant change ($p > 0.05$) in expression (Figure 42). There was a greater than 80-fold increase ($p < 0.05$) in the expression of the megakaryocytic specific gene, *PF4*, when cells were exposed to PMA (Figure 43). This increase was reduced by over 25% ($p < 0.05$) when treated simultaneously with anagrelide (Figure 44). There was a slight increase in *PSTPIP2* in PMA-induced cells, although this was not significant ($p > 0.05$) (Figure 45). Similar to PF4, there was a significant ($p < 0.05$) 25% decrease in expression when cells were treated concomitantly with anagrelide (Figure 46).

Induced differentiation of HEL cells by PMA does not alter the expression of *GATA1*, although the levels of the three GATA1 target genes are elevated, significantly in the case of *GPIX* and *PF4*. It must also be noted that while *GATA1* expression during differentiation was unchanged when exposed to anagrelide, both *PF4* and *PSTPIP2* decreased. The downregulation of these genes in response to anagrelide may be due either to post-translational modifications to GATA1 or GATA1-independent mechanisms. As *GPIX* is unaffected by anagrelide, this may indicate that GATA1-independent mechanisms are likely to be responsible for the activity of anagrelide in this cell line.



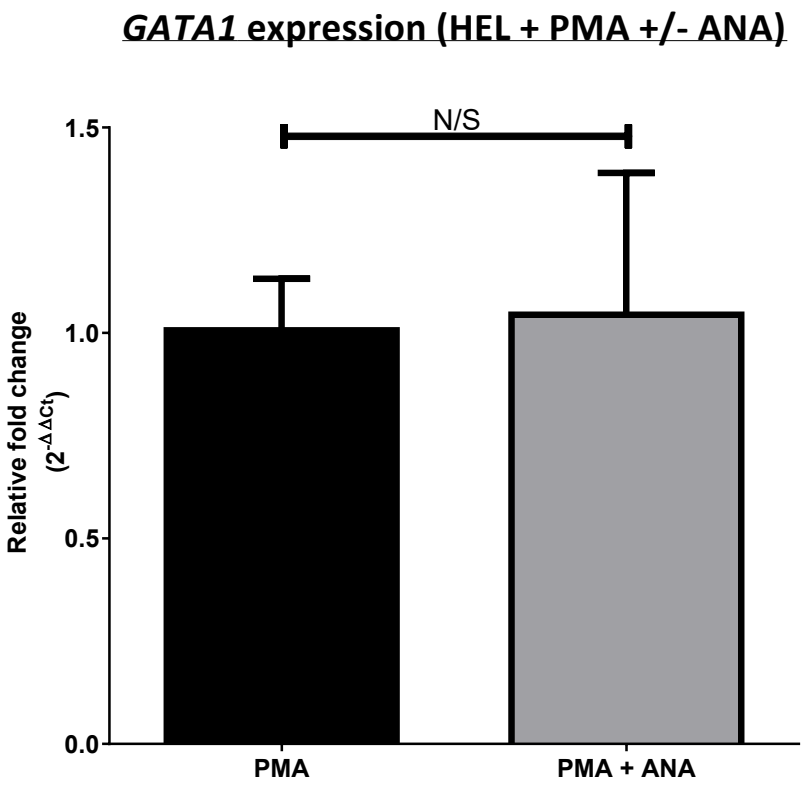


Figure 39: HEL cells treated with PMA to induce differentiation. No difference in gene expression was observed for *GATA1* (t-test; $p > 0.05$). Experiment carried out three independent times (n = 3 ± SD).

Figure 40: HEL cells treated with PMA to induce differentiation in the presence of anagrelide or control (DMSO). No difference in gene expression was observed for *GATA1* (t-test; $p > 0.05$). Experiment carried out three independent times (n = 3 ± SD).

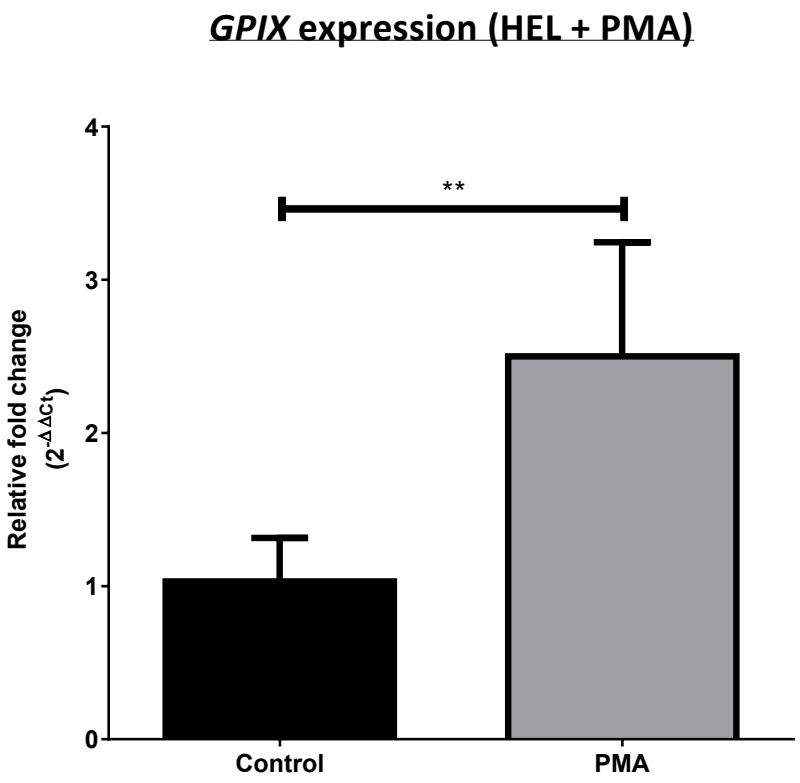


Figure 41: HEL cells treated with PMA to induce differentiation. A significant upregulation in gene expression was observed for *GPIX* (t-test; $p < 0.05$). Experiment carried out in triplicate (n = 3 ± SD).

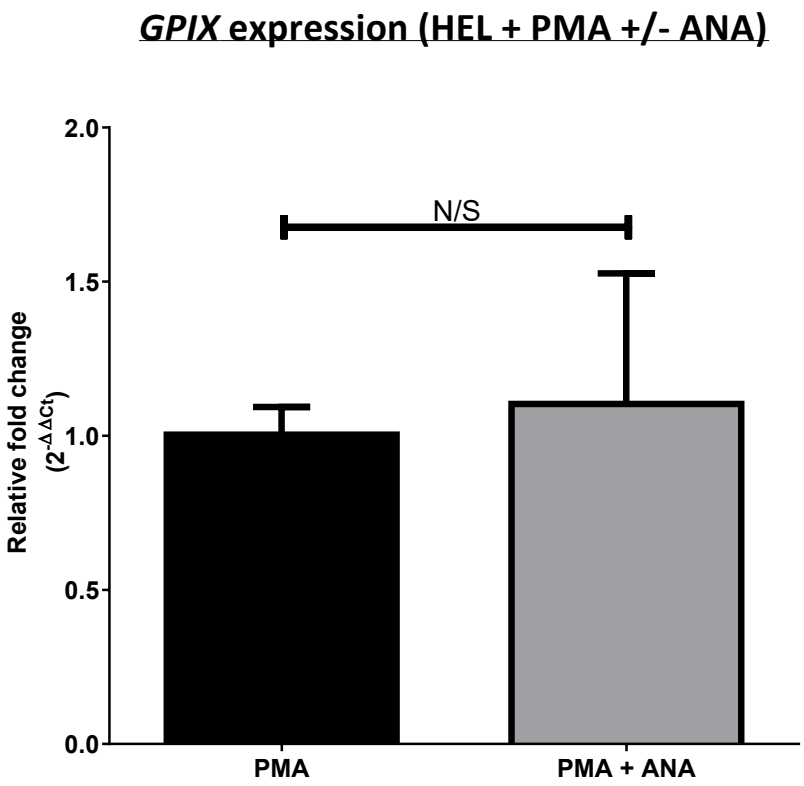


Figure 42: HEL cells treated with PMA to induce differentiation in the presence of anagrelide or control (DMSO). No difference in gene expression was observed for *GPIX* (t-test; $p > 0.05$). Experiment carried out on three independent time points (n = 3 ± SD).

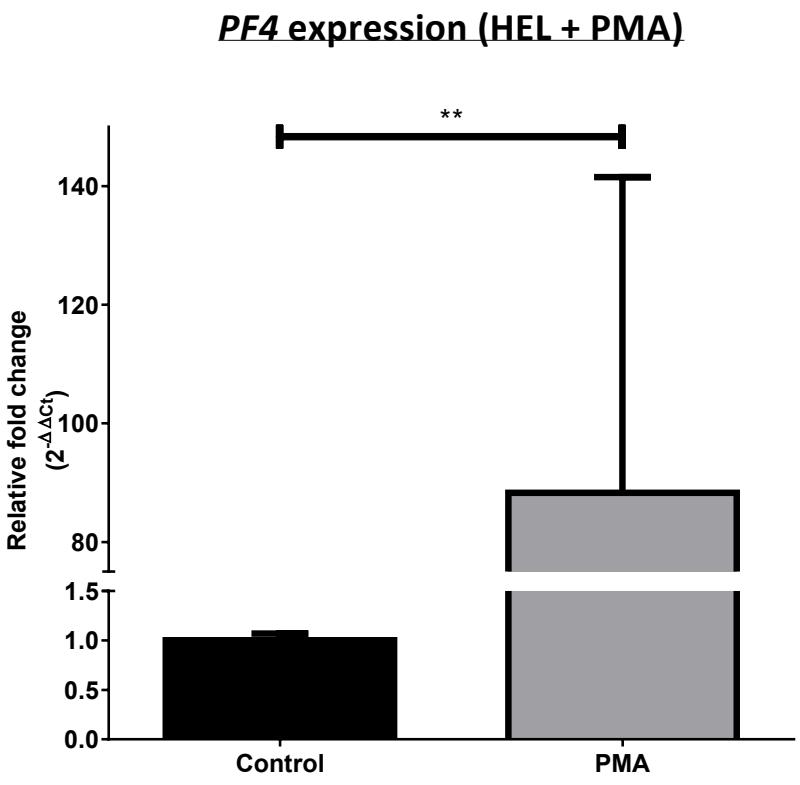


Figure 43: HEL cells treated with PMA to induce differentiation. A significant upregulation in gene expression was observed for *PF4* (t-test; $p < 0.05$). Experiment carried out three independent times (n = 3 ± SD).

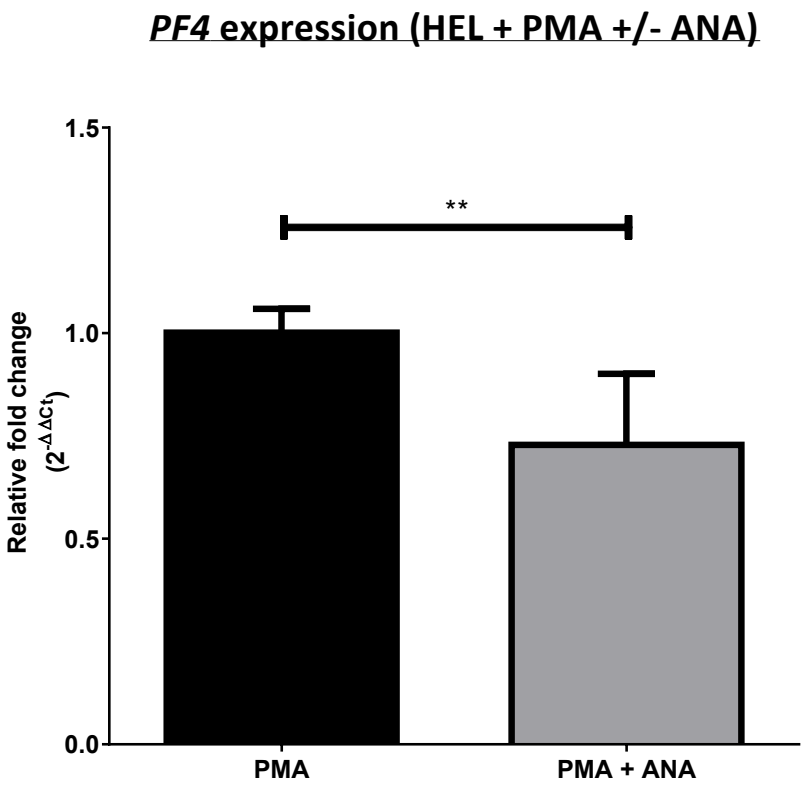


Figure 44: HEL cells treated with PMA to induce differentiation in the presence of anagrelide or control (DMSO). *PF4* expression was significantly downregulated when treated with anagrelide (t-test; $p < 0.05$). Experiment carried out three times (n = 3 ± SD).

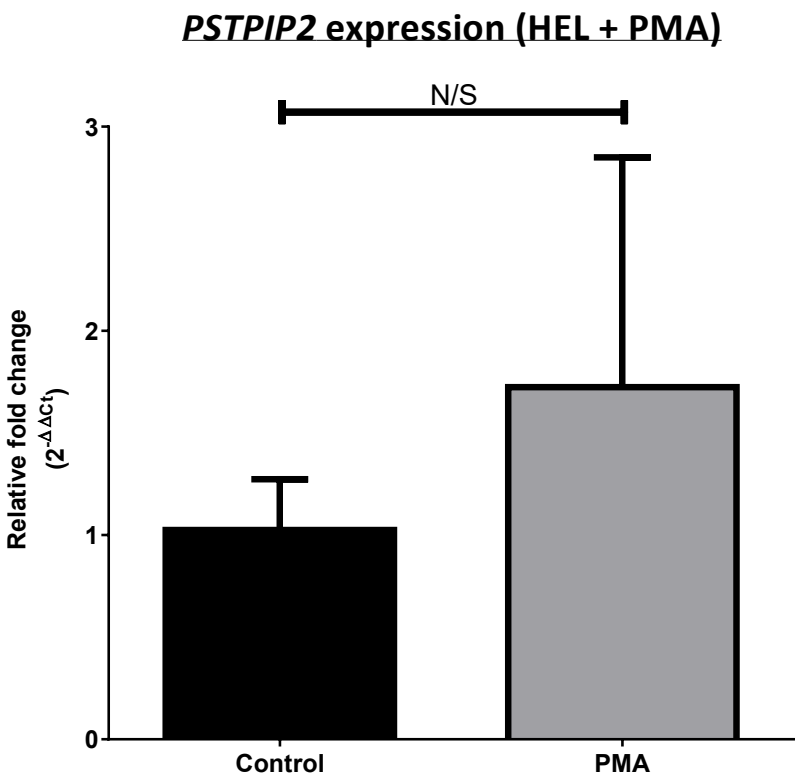


Figure 45: HEL cells treated with PMA to induce differentiation. No difference in gene expression was observed for *PSTPIP2* (t-test; $p > 0.05$). Experiment carried out three times (n = 3 ± SD).

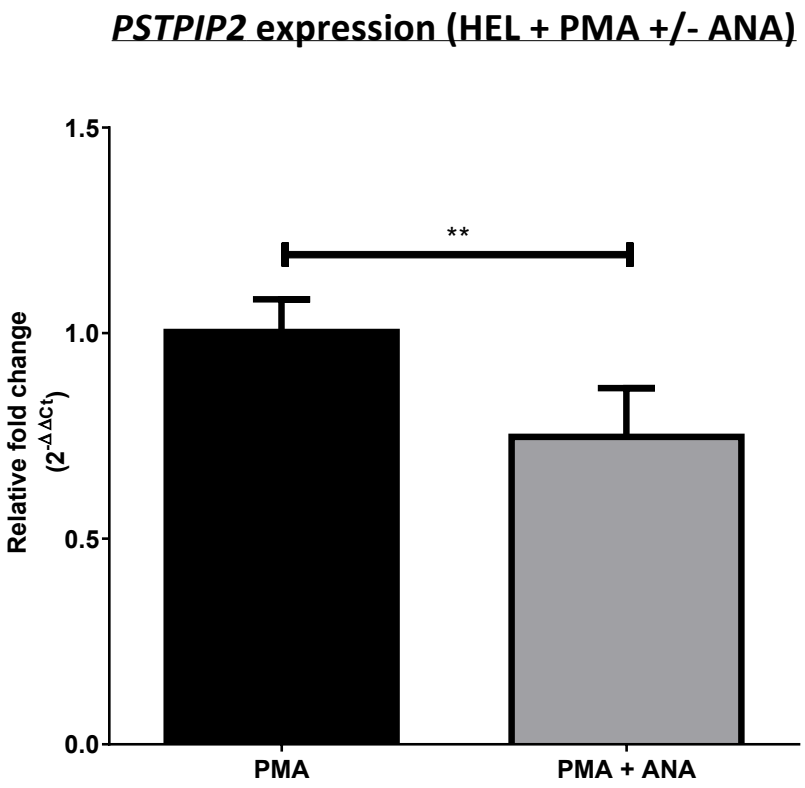


Figure 46: HEL cells treated with PMA to induce differentiation in the presence of anagrelide or control (DMSO). *PSTPIP2* expression was significantly downregulated when treated with anagrelide (t-test; $p < 0.05$). Experiment carried out three times (n = 3 ± SD).

4.5: Discussion

Cell proliferation in K562, HL60, SET2 and UKE1 cell lines (Figure 26 - Figure **29**) as measured by the MTS assay was unchanged by anagrelide treatment. This was expected, since the drug is not believed to be cytotoxic, unlike other therapies (e.g. hydroxyurea) used to control platelet counts in ET patients (Hong and Erusalimsky, 2002). Interestingly, the HEL cell line did respond significantly (Figure 25) to anagrelide with an IC_{50} of 100 µM. It is notable that a drug whose main mode of effect is in halting differentiation (Hong *et al.*, 2006) should also prevent proliferation at high concentrations. Unfortunately, it was not possible to determine the concentration at which total inhibition of proliferation occurred, as the stock dilution and DMSO toxicity prevented concentrations greater than 100 µM.

Reductions in proliferation in HEL were confirmed using trypan blue exclusion assays, viability was also reduced. This suggests that anagrelide does have a cytotoxic effect, at least in the HEL cell line.

Following on from cell proliferation experiments, propidium iodide staining was used to determine the stage of the cell cycle at which anagrelide inhibited proliferation in HEL. Cell cycle studies showed that anagrelide resulted in a G0/G1 block in the HEL cells affected, with corresponding decreases in cells in S and G2/M stages. No changes were observed in K562. Megakaryocyte development is characterised by endomitosis, a change in the normal process of mitosis (Wen *et al.*, 2011). This is where DNA is repeatedly duplicated but cytoplasmic duplication does not occur, leading to an increase in cell ploidy. Cell cycle kinetics are altered with a shorter G1 phase, normal S phase, short G2 phase and a truncated mitotic phase (Wen *et al.*, 2011). Therefore, regulation of key cell cycle checkpoints is critical for normal megakaryopoiesis. Anagrelide is known to reduce both diameter and ploidy of megakaryocytes (Espasandin *et al.*, 2015), suggesting a possible role in regulating cell cycle genes involved in endomitosis. Upregulation of Cyclin E, critical for progression through G1 phase, in transgenic animals results in polyploidisation (Eliades *et al.*, 2010), and knockout results in defective megakaryocyte endoreplication (Geng *et al.*, 2003). Another cyclin protein family member, cyclin D1,

is responsible for G1/S transition (Sun *et al.*, 2001). Overexpression of cyclin D1 leads to increased polyploidisation (Sun *et al.*, 2001). In addition, cyclin D1 has been demonstrated to be a direct GATA1 target in megakaryocytes but not erythroid cells (Muntean *et al.*, 2007). The role of anagrelide in inhibiting cyclic AMP phosphodiesterase 3 (cAMP PDE3) may also be a factor. Inhibition of megakaryopoiesis by cAMP has been shown to be controlled by downregulation of E2A and its target CDKN1A/p21, which is negatively regulated by anagrelide (Rubinstein *et al.*, 2012). Research using other PDE3 inhibitors does not show any changes in megakaryocyte maturation *in-vitro* (Espasandin *et al.*, 2015).

These results suggest that anagrelide does have an effect on cell cycle progression, although it is uncertain as to whether these mechanisms are responsible for its anti-platelet activity or the reasons why it is only observed with the HEL cell line.

It is noted that the concentration used in cell cycle experiments (1 µM) was typically greater than the plasma concentration of the drug in patients. This is approximately 5-50 ng/mL (Mazur *et al.*, 1992), or 20-200 nM. There was significant reduction in proliferation from 10 nM (Figure 25) and viability at 100 nM (Figure 34), which are within or lower than the therapeutic dose range.

No changes in gene expression, including *GATA1*, were observed when K562, HEL or SET2 were treated with anagrelide. Anagrelide has previously been demonstrated to affect the expression of several genes, including *GATA1, FOG, FLI1* and *NFE2*, during TPO-induced megakaryopoiesis in cultured UT-7/mpl or primary haematopoietic cell lines (Ahluwalia *et al.*, 2010), but has no reported impact on JAK2 phosphorylation status. This was a key rationale behind using this drug to probe changes in *GATA1* and its role in essential thrombocythaemia. It is unknown whether the mechanism for the inhibition for proliferation in HEL cells is the same as that observed in megakaryocyte differentiation. The antiproliferative activity of anagrelide on this cell line is therefore not directly linked to modulation of *GATA1* expression.

To investigate whether *GATA1* expression was altered by anagrelide during megakaryopoiesis, the phorbol ester, PMA, is a protein kinase C activator and induces p21 expression in a p53 independent manner (Park *et al.*, 2001). Elevated levels of cyclic AMP induced by anagrelide inhibits megakaryopoiesis by targeting E2A-p21 transcriptional axis (Rubinstein *et al.*, 2015). PMA was used to induce megakaryocytic changes in the HEL cell line. When exposed to PMA with and without anagrelide, there was no difference in proliferation or cell adhesion to the plate surface (Figure 38). Experiments using CD34+ haematopoietic cells induced to differentiate with TPO in the presence of anagrelide have shown reduction in megakaryocyte numbers but not in mononuclear cells (McCarty *et al.*, 2006).

Inducing differentiation using PMA did not result in any change in the expression levels of *GATA1* and this was unaltered when anagrelide was used concomitantly. The previously mentioned study by (Ahluwalia *et al.*, 2010) showed GATA1 increasing when primary haematopoietic cells were cultured under megakaryocytic differentiation conditions, this was reduced when treated with anagrelide. This is likely to be a result of key differences in the experimental setup of both studies. The results presented here used PMA to induce differentiation in transformed immortalised HEL cells as opposed to TPO in primary haematopoietic cells. *GATA1* has multifunctional roles during haematopoiesis and its expression levels are linked to each particular stage of differentiation (Ferreira *et al.*, 2005).

The presence of anagrelide (Figure 27) resulted in reduced expression of two megakaryocytic genes, *PF4* and *PSTPIP2* but not in *GATA1* or the megakaryocytic specific *GPIX*. The decrease in *PF4* is noteworthy since it acts as a negative regulator of megakaryopoiesis in both *in-vitro* and *in-vivo* models (Lambert *et al.*, 2007) but has also been shown to induce megakaryopoiesis and platelet accumulation in lung-tumour bearing mice (Pucci *et al.*, 2016). In ET patients, levels of PF4 released from platelets are higher than controls and treatment with anagrelide resulted in a significant reduction which was not observed with either HU or IFN-α (Cacciola *et al.*, 2004). Recently, gene expression analysis on CD34+ cord blood derived cells showed that anagrelide was responsible for

downregulating genes associated with megakaryocyte proteins but had no effect on *GATA1* (Sakurai *et al.*, 2016), reflecting the results found here.

The clinical efficacy of anagrelide in reducing platelet counts in patients with ET is known, but the full molecular mechanism is not well-understood. These results, in a cell line responsive to anagrelide, indicate that *GATA1*, which is overexpressed in ET, is not a direct target of anagrelide activity. Downstream targets of *GATA1* involved in megakaryopoiesis are affected by anagrelide during PMA-induced megakaryocytic differentiation indicating that direct transcriptional control of *GATA1* is unlikely to be responsible but *GATA1*-independent mechanisms or post-translational modifications may play a role in the disease. This may also be a potential mechanism for the activity of anagrelide in ET patients and a possible target for novel therapies.

CHAPTER 5: Molecular mechanisms of JAK2 dysregulation

5.1: Introduction

Mutations in the non-receptor tyrosine kinase, *JAK2*, have been identified in over 95% of PV and between 50-60% of ET and MF cases (O'Sullivan and Harrison, 2017). Constitutive activation of the JAK/STAT pathway is a hallmark of myeloproliferative neoplasms, yet it is not fully understood how JAK/STAT pathway dysregulation gives rise to phenotypically different disorders in PV, ET, and MF, or the factors which determine disease progression and leukaemic transformation.

Ruxolitinib is a JAK1/JAK2 inhibitor which prevents activation of JAK/STAT signalling by blocking the ATP binding site on the kinase domain of JAK2 (Ostojic *et al.*, 2011). It is not specific for the $JAK2^{V617F}$ mutated form and has activity against $JAK2^{WT}$ (Vandenberghe, 2012). Clinical trials have demonstrated its efficacy in reducing some of the symptoms associated with MPNs, including splenomegaly, as well as an improvement in overall survival (Verstovsek *et al.*, 2012a).

While gene expression analysis is an invaluable tool in studying the effects of JAK/STAT pathway dysregulation, mRNA levels do not always accurately correlate with expressed protein levels (Greenbaum *et al.*, 2003). Post-translational modifications can dynamically alter the function, activity, and half-life of proteins in a manner that is not reflected in transcriptional analysis. These modifications may involve the addition of a chemical group and are reversible, e.g., acetylation/deacetylation and phosphorylation, or are irreversible, e.g., proteolysis. A global quantitative proteomic analysis coupled with investigation of key post-translational modifications is therefore a complementary method of studying JAK/STAT signalling dysregulation.

5.2: Aims

The following experiments will aim to characterise the effect of JAK2 inhibition (using ruxolitinib) on MPN patient derived cells. Model $JAK2^{V617F}$ and $JAK2^{WT}$ cell lines will also be used to study the impact on cell proliferation, STAT phosphorylation and signalling, and changes to the global proteome.

5.3: Methods

Methods are described in brief here. For full descriptions refer to Chapter 2.

Experiments using model *JAK2*^{V617F} and *JAK2*^{WT} cell lines:

MTS Assays: Cells (K562, HL-60, HEL, SET2 and UKE1) were incubated for 72 hours in the presence of inhibitor or vehicle control (DMSO). After incubation, 20 µL of MTS (Promega) with PMS (Sigma) (50:1) solution was added to 100 µL of cells in culture media. Cells were incubated for 2 – 4 hours and absorbance at 490 nm was measured. Control wells were treated with 10% Triton-X100 (Sigma) 20 minutes prior to addition of PMS for complete cell death.

Cell Cycle Analysis: Cell lines (K562 and HEL) were serum-starved overnight prior to treatment with ruxolitinib (Selleckchem). After 48 hours of treatment, cells were collected by centrifugation and washed with ice-cold PBS. Cell fixation and propidium iodide staining were performed as per published protocols (Darzynkiewicz and Juan, 2001). Cell fluorescence was measured on a FACSVerse (BD Biosciences) and analysis carried out using FlowJo VX software (Treestar Inc).

Western Blotting: Cell culture and drug treatment was performed as previously described in Chapter 2. Total protein (10 mg/µL) was obtained from cells lysed in mRIPA buffer and run on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with primary antibodies for pSTAT1, pSTAT3, pSTAT5, STAT1, STAT3, STAT5 and GAPDH. Secondary antibodies (anti-rabbit IgG) with a HRP conjugate were used for detection with a chemiluminescent reagent (Thermo ECL).

Experiments using cells from donor patients:

Colony-Forming-Cell (CFC) Assays: Peripheral blood mononuclear cells from donors were isolated using MethoCult media containing 0.1 µM EPO according to the Stemcell protocol for CFC assays (Stemcell Technologies). Cells were plated (2 x 10^5 cells per dish). Plates were examined after 10 days

and enumerated after 14 days in culture. Individual BFU-E colonies were trypsinised and taken from the plates, washed two times with PBS and the cell pellet stored at -80ºC.

Experiments using model cell lines and patient samples:

Quantitative Proteomics: 8-plex iTRAQ labelling was used to identify differentially expressed proteins in ruxolitinib treated HEL and SET2 cell lines, as well as cells from MPN patients treated o/n with ruxolitinib. Digested and labelled peptides were separated using a NanoLC with electrospray ionisation coupled to a Triple TOF 6600 mass spectrometer. The instrument was operated in data dependent mode. Peptides with multiple charges (2^+ to 4^+) with a charge to weight ratio (*m/z*) between 400 and 1600 were selected for MS/MS. Output files were processed using Protein Pilot software (version 5.0.1). Data analysis was carried out using Microsoft Excel, Cytoscape v3.5.0 with ClueGO plugin and GraphPad Prism v 6.0.

5.4: Results

5.4.1: Ruxolitinib selectively inhibits the growth of *JAK2^{V617F}* mutated cell lines

Cell lines exposed to increasing concentrations of the tyrosine kinase inhibitor, ruxolitinib, showed different growth responses depending on whether the *JAK2^{V617F}* mutation was present. All *JAK2^{V617F}* cell lines (Figure 47 - Figure 49) demonstrated reduced proliferation after 72 hours exposure to the drug. In contrast, there was no effect on *JAK2^{WT}* cell lines (Figure 50 and Figure 51), indicating that the increased JAK2 activity in the mutant cell lines was responsible for their sensitivity to JAK inhibition by ruxolitinib. IC_{50} values in the *JAK2^{V617F}* expressing cell lines do not appear to be directly related to loss of heterogeneity. SET2, which expresses both mutant and wild-type JAK2, had a similar IC_{50} (132 nM) to the homozygous UKE1 cell line (109 nM), whereas HEL was 565 nM. Both SET2 and UKE1 cell lines were derived from patients with a previous history of ET, but HEL cell line had no recorded MPN or MDS prior to AML (Martin and Papayannopoulou, 1982, Quentmeier *et al.*, 2006). It is not known whether this may influence sensitivity to JAK/STAT targeting by ruxolitinib.

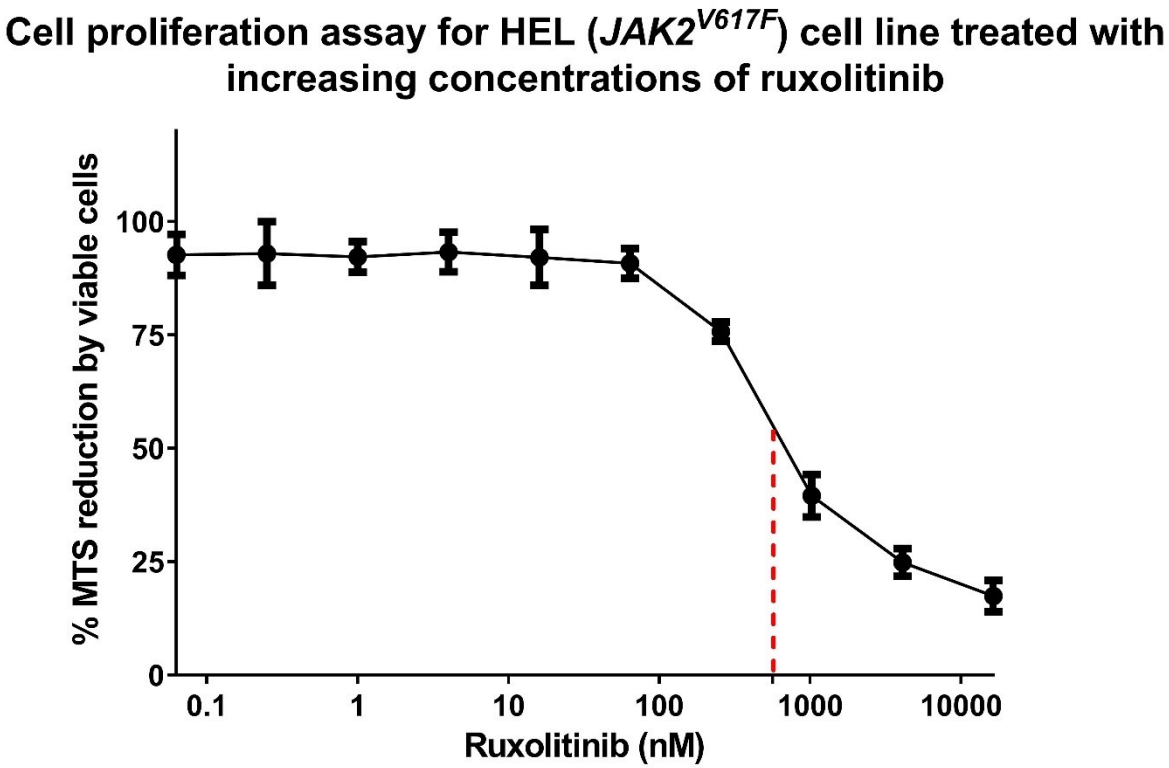


Figure 47: Cellular proliferation assay in the *JAK2*^{V617F} cell line, HEL + ruxolitinib. Viability, measured by MTS reduction, was reduced in response to increasing concentrations of ruxolitinib. IC_{50} value determined using GraphPad Prism software: 565 nM (n = 3 ± SD).

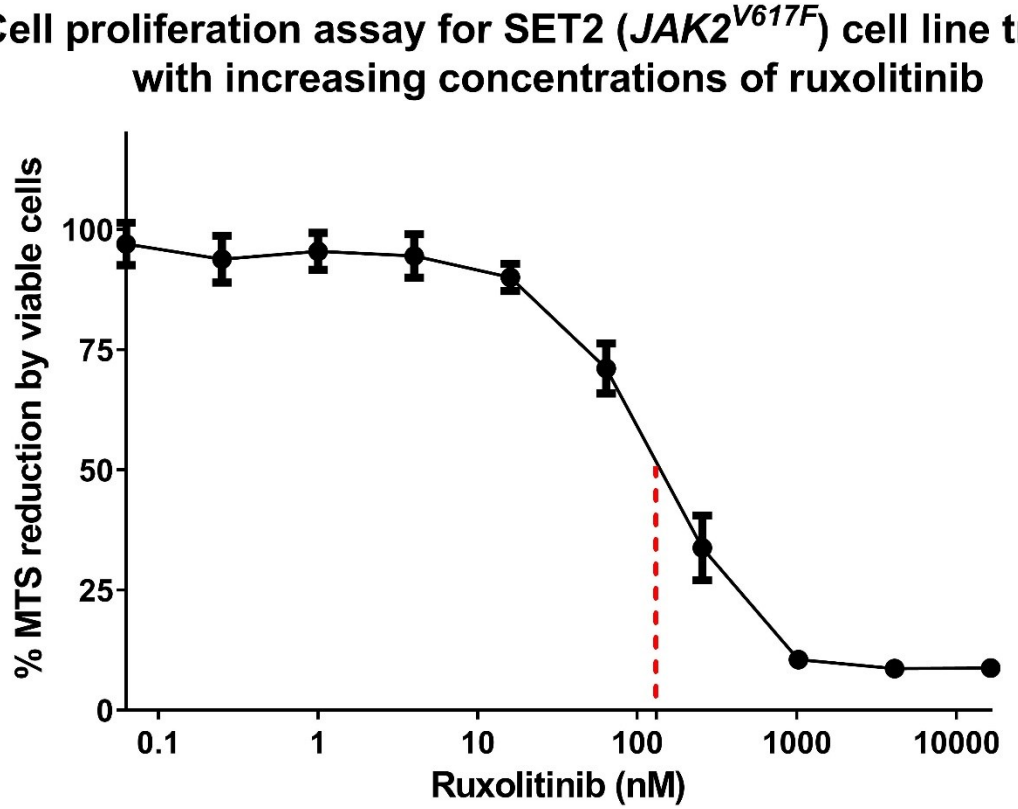


Figure 48: Cellular proliferation assay in the *JAK2*^{V617F} cell line, SET2 + ruxolitinib. Viability, measured by MTS reduction, was reduced in response to increasing concentrations of ruxolitinib. IC_{50} value determined using GraphPad Prism software: 132 nM (n = 3 ± SD).

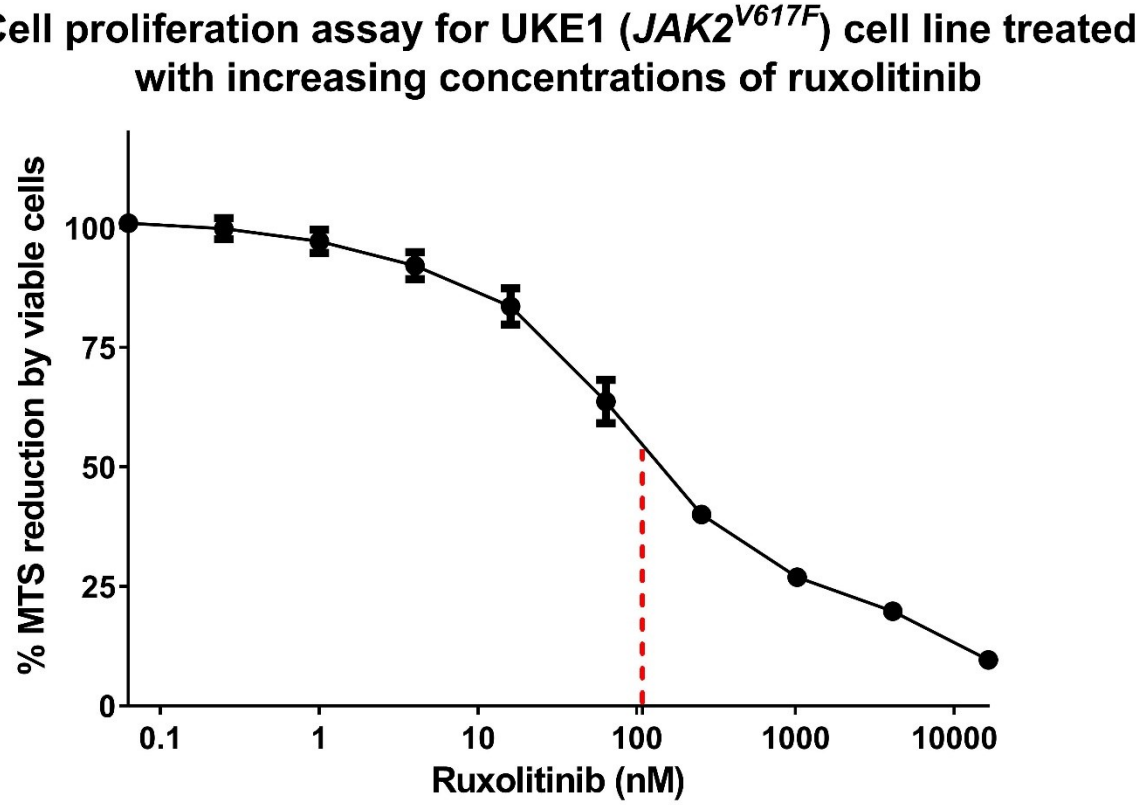


Figure 49: Cellular proliferation assay in the *JAK2*^{V617F} cell line, UKE1 + ruxolitinib. Viability, measured by MTS reduction, was reduced in response to increasing concentrations of ruxolitinib. IC_{50} value determined using GraphPad Prism software: 109 nM (n = 3 ± SD).

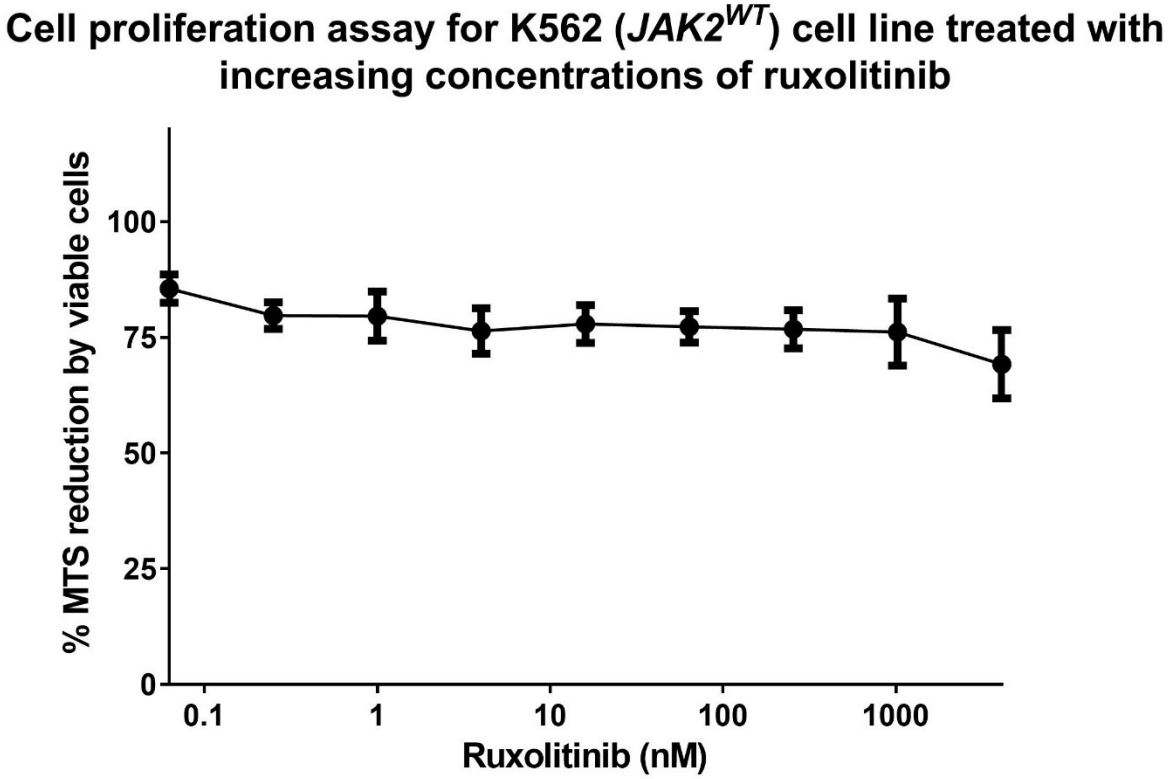


Figure 50: Cellular proliferation assay in the *JAK2*^{WT} cell line, K562 + ruxolitinib. Cell viability, measured by MTS reduction, was not affected by ruxolitinib treatment. There was no significant difference (t-test; $p > 0.05$) in proliferation at highest tested concentration (10,000 nM) (n = 3 ± SD).

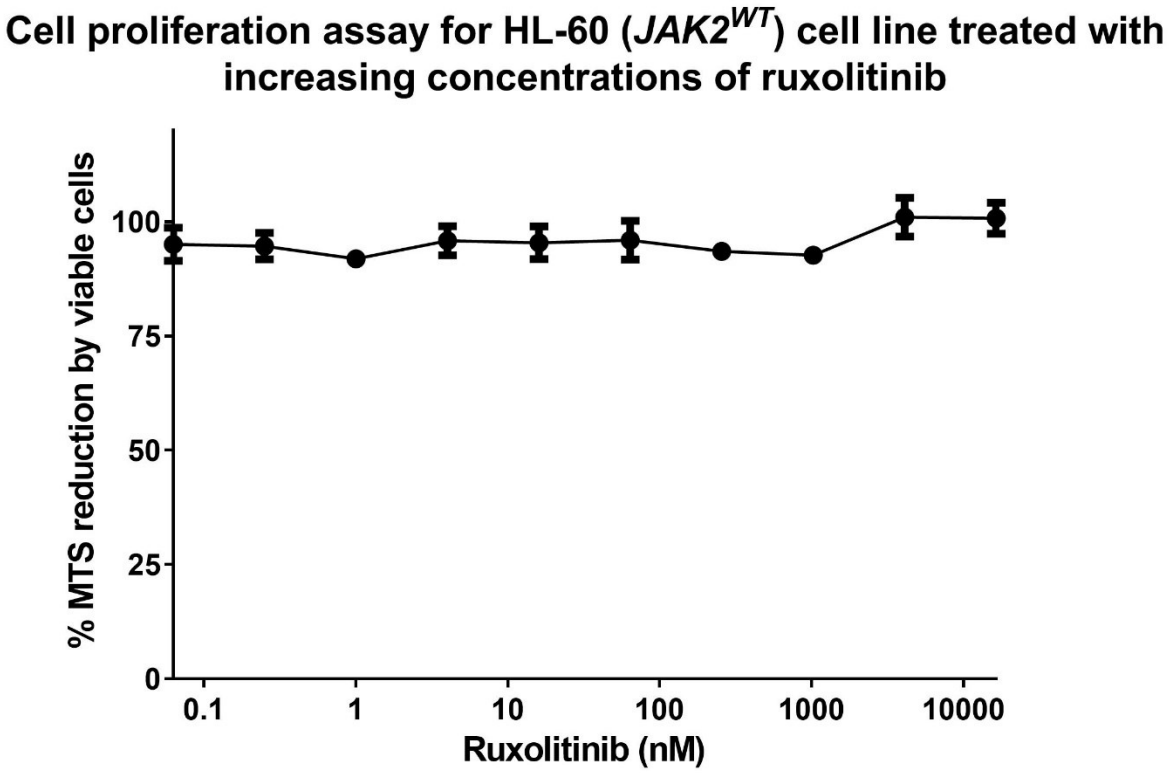


Figure 51: Cellular proliferation assay in the *JAK2*^{WT} cell line, HL60 + ruxolitinib. Cell viability, measured by MTS reduction in HL60, was not affected by ruxolitinib treatment. No IC_{50} concentration determined. There was no significant difference (t-test; $p > 0.05$) in proliferation at highest tested concentration (10,000 nM) (n = 3 ± SD).

5.4.2: G0/G1 increase in HEL cells treated with ruxolitinib

The proportion of K562 and HEL cells in each stage of the cycle stage was measured in response to ruxolitinib treatment (1 µM) over 72 hours. No differences were observed between the vehicle control and 1 µM RUX-treated K562 cells (Figure 52 and Figure 53). Chi-square test results: $\chi^2 = 3.656$, df = 3, $p > 0.05$.

There was an increase in cells in the sub G0 (t-test; $p < 0.05$) and G0/G1 (t-test; $p < 0.05$) phases after HEL cell were treated with ruxolitinib, as well as a corresponding decrease in S (t-test; $p < 0.05$) and G2/M (t-test; $p < 0.05$) (Figure 54 and Figure 55). Chi-square test results: $\chi^2 = 420.1$, df = 3, $p < 0.05$.

These results reflect the findings from the previous MTS experiments showing cells carrying a *JAK2*^{V617F} mutation (Figure 47 - Figure 49) were more sensitive to JAK/STAT inhibition than those with wild type *JAK2* (Figure 50 and Figure 51). The mechanism for inhibited proliferation by ruxolitinib in *JAK2* mutated cells is associated with a G0/G1 block by ruxolitinib. This is consistent with its inhibition of JAK/STAT signalling and its impact on the cell cycle.

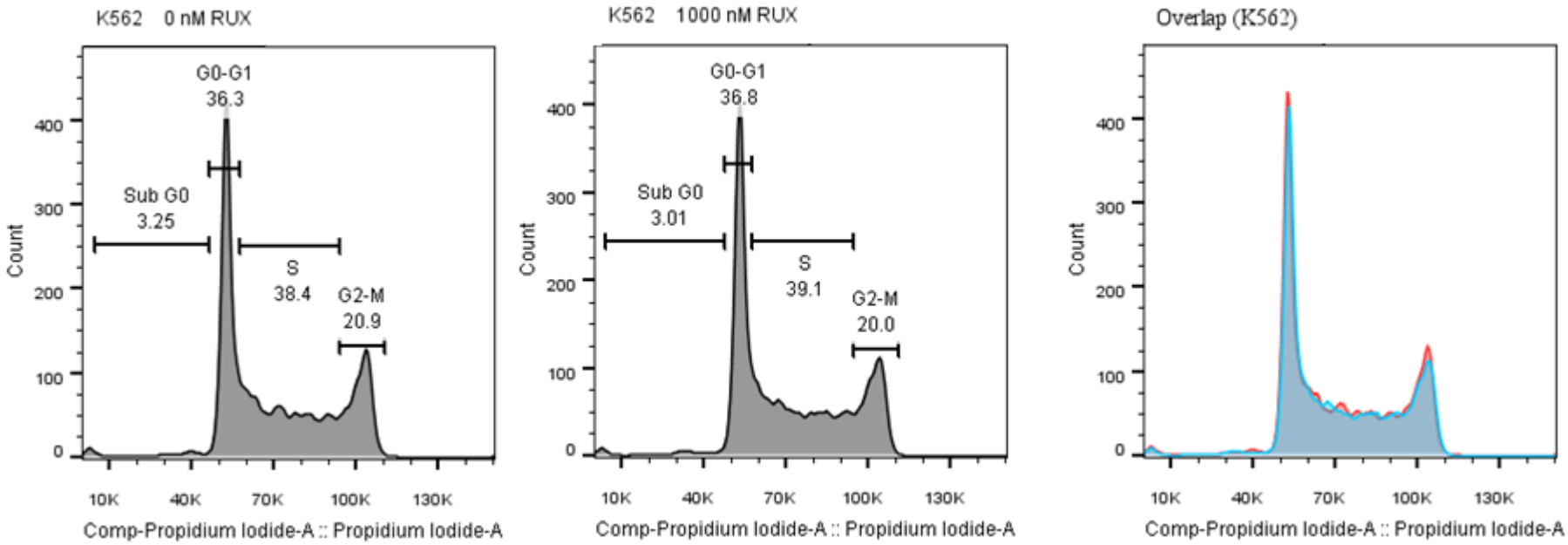


Figure 52: Effect of ruxolitinib on cell cycle in K562 cells. Representative experiment shown in the figure (FlowJo Software), experiment was carried out in triplicate. No difference was observed in the proportion of K562 cells in each stage of the cell cycle ± ruxolitinib.

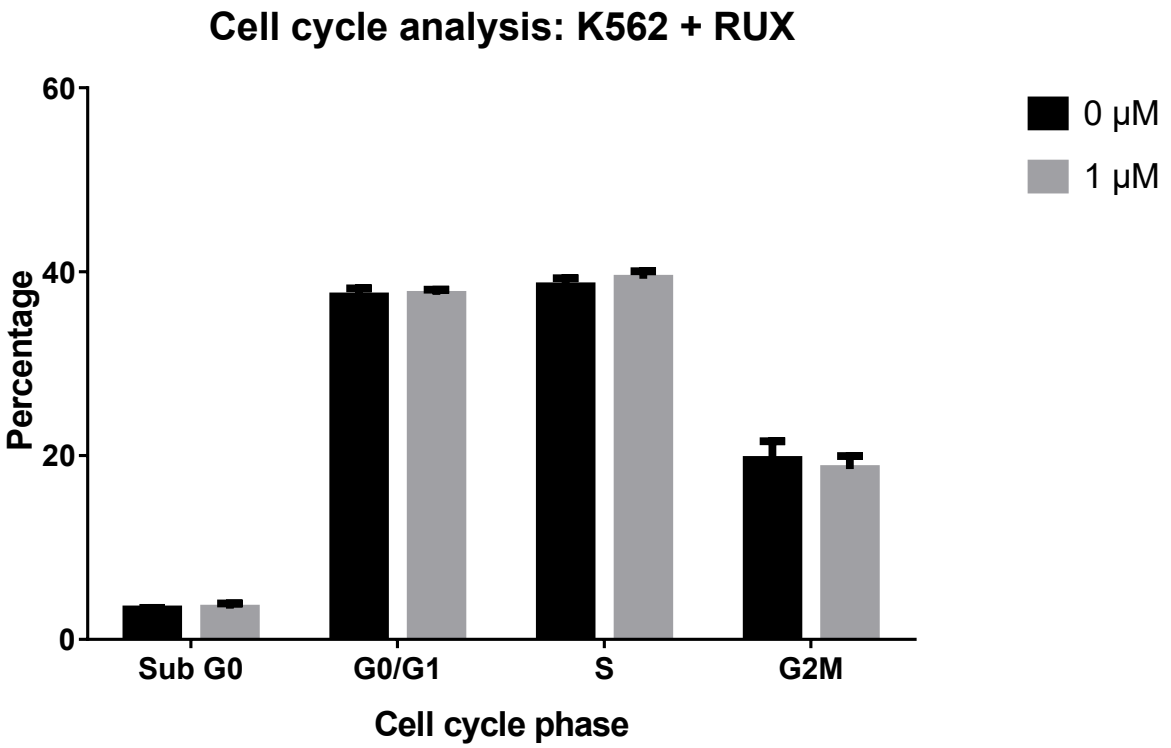


Figure 53: Cell cycle analysis of K562 cells treated with ruxolitinib for 48 hours. Bars show average percentage cells in each phase of the cell cycle with standard deviation (n = 3 ± SD). Ruxolitinib did not have any significant effect (t-test; $p > 0.05$) on cell cycle phases in the K562 cell line.

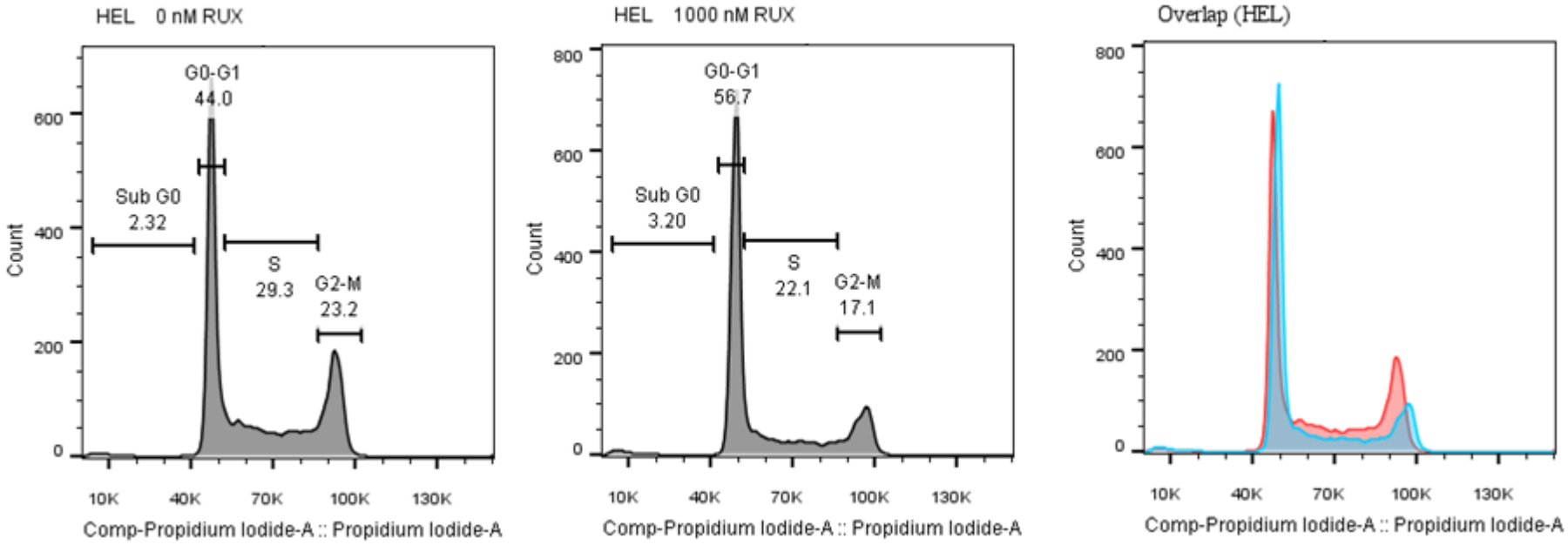


Figure 54: Effect of ruxolitinib on cell cycle in HEL cells. Representative experiment shown in the figure (FlowJo Software), experiment was carried out in triplicate. An increase in the proportion of cells in G0/G1 was observed in response to ruxolitinib treatment, along with a corresponding decrease in the number of cells in S and G2/M phases.

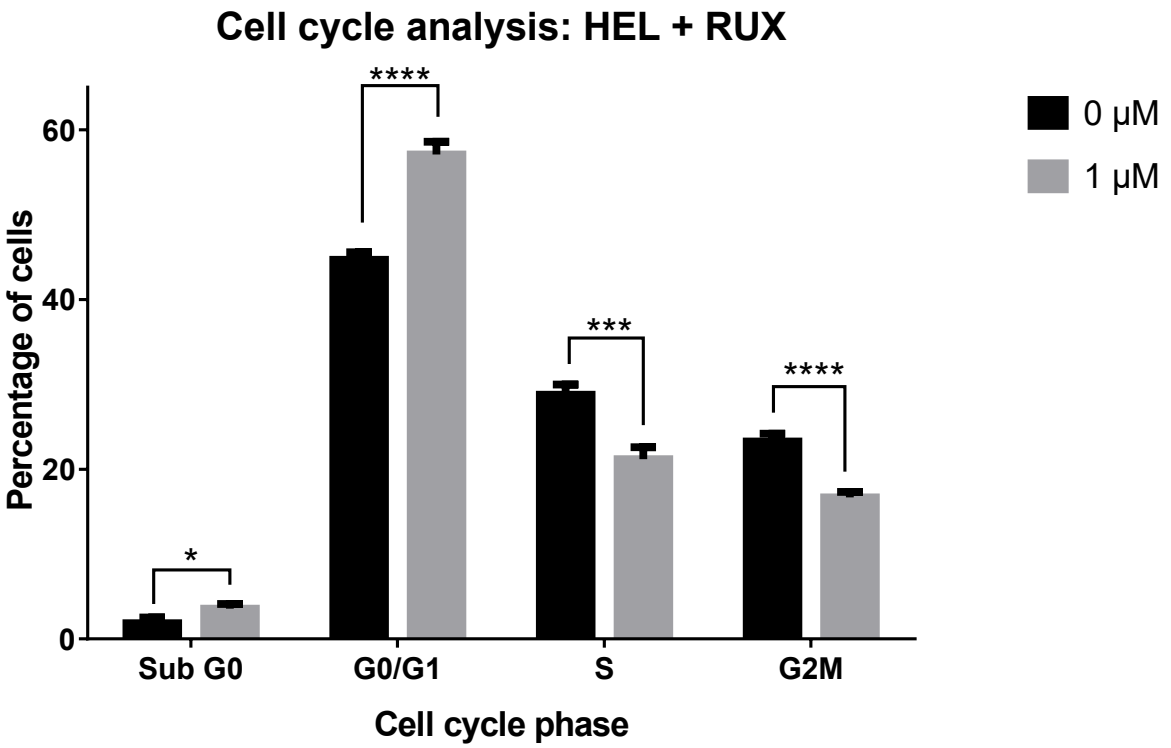


Figure 55: Cell cycle analysis of HEL cells treated with ruxolitinib for 48 hours. Bars show average percentage cells in each phase of the cell cycle with standard deviation (n = 3 ± SD). In HEL treated cells, a significant increase ($p < 0.05$) was observed in number of cells in Sub G0 phase (t-test; $p < 0.05$) and G0/G1 phase (t-test; $p < 0.05$) on ruxolitinib treatment. Conversely significant decreases were measured in cell numbers entering S (t-test; $p < 0.05$) and G2/M (t-test; $p < 0.05$) phases.

5.4.3: Ruxolitinib reduces phosphorylation of STAT3 and STAT5 in *JAK2^{V617F}* cell lines and increases phosphorylation of JAK2

Constitutive activation of the JAK-STAT pathway is a characteristic feature of MPNs. Over-activation of this pathway, as indicated by STAT3 and STAT5 phosphorylation, is seen in the model MPN cell lines HEL and SET2 when compared to the CML cell line, K562 (Figure 56). As a result of treatment with the JAK2 inhibitor, ruxolitinib, a dose-dependent inhibition of downstream signalling pathways was indicated by a reduction in phosphorylation of STAT3 and STAT5. The greatest effect was observed with STAT5 phosphorylation, with 1.0 µM ruxolitinib enough to completely knockdown pSTAT5 in HEL and SET2. The reduction in STAT3 phosphorylation was more modest in HEL and SET2, although lower than observed in the *JAK2^{WT}* cell line, K562. Conversely, the level of JAK2 phosphorylation was enhanced with increasing doses of ruxolitinib in the *JAK2^{V617F}* cell lines. Phosphorylated JAK2 was barely detectable in the vehicle control samples for HEL and SET2, and any of the K562 samples (control and treated).

Ruxolitinib indirectly affects phosphorylation levels of the key signalling proteins, STAT3 and STAT5 by targeting JAK2. The drug is an ATP mimetic, which competes for binding at the kinase domain of JAK2. This action renders JAK inaccessible for recruitment, phosphorylation and dimerisation of STAT proteins. These results demonstrate that ruxolitinib, despite increased catalytic activity in the kinase domain characterised by increased phosphorylation at Tyr1007 on JAK2, does not result in phosphorylation of downstream JAK/STAT signalling pathway targets. The long-term effects, such as resistance like that seen with other tyrosine kinase inhibitors, of constant JAK activation are not fully understood but other studies have suggested that it may contribute towards acquired persistence to drug therapy. Ruxolitinib treatment did not affect the total protein levels of either STAT3 or STAT5.

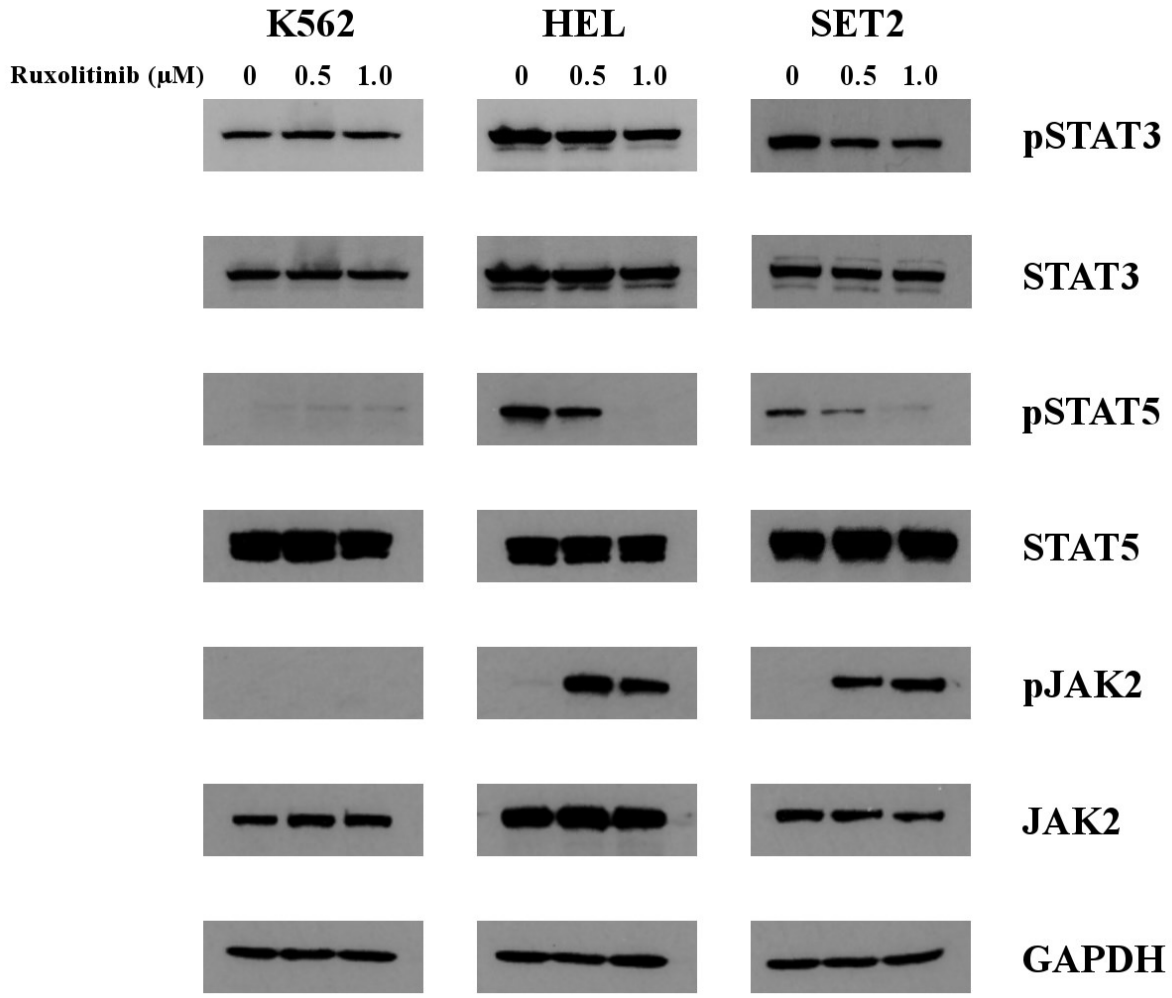


Figure 56: Western blots of phosphorylated and total STAT3, STAT5, and JAK2 proteins in K562 ($JAK2^{WT}$), HEL, and SET2 ($JAK2^{V617F}$) after 24 hours in response to ruxolitinib treatment. Both phosphorylated STAT3 and STAT5 decreased with increasing concentrations of ruxolitinib in $JAK2^{V617F}$ cell lines. Phosphorylated levels of JAK2 increased in in $JAK2^{V617F}$ cell lines when exposed to ruxolitinib.

5.4.4: Ruxolitinib treatment of peripheral blood mononuclear cells from patient samples results in a decrease in numbers of myeloid and erythroid progenitor cells

All four patients had elevated levels of haemoglobin, above the WHO diagnostic threshold for PV (> 165/160 g/L in men/women). ET samples had increased levels of circulating platelets (> 455 x 10^9 /L). Patients were not receiving any JAK inhibitor therapy, which could affect results for experiments where isolated PBMCs were treated with ruxolitinib. PBMCs from patients with high levels of either haemoglobin or platelet counts, and control patient samples, were treated with either 100 nM or 250 nM RUX in methylcellulose culture media for 14 days. Numbers of all colony types were significantly lower with 100 or 250 nM ruxolitinib treatments (Figure 58 - Figure 60). Relative reduction in all colony types was lowest for the control sample treated with ruxolitinib, followed by ET, and then PV groups. In addition to reduced numbers of colonies, ruxolitinib treatment resulted in smaller BFU-E colony size in culture (Figure 57). Sensitivity to ruxolitinib treatment in PBMCs does not appear to be related to the presence of a *JAK2* mutation or increased haematopoietic markers (haemoglobin or platelets) in PV and ET.

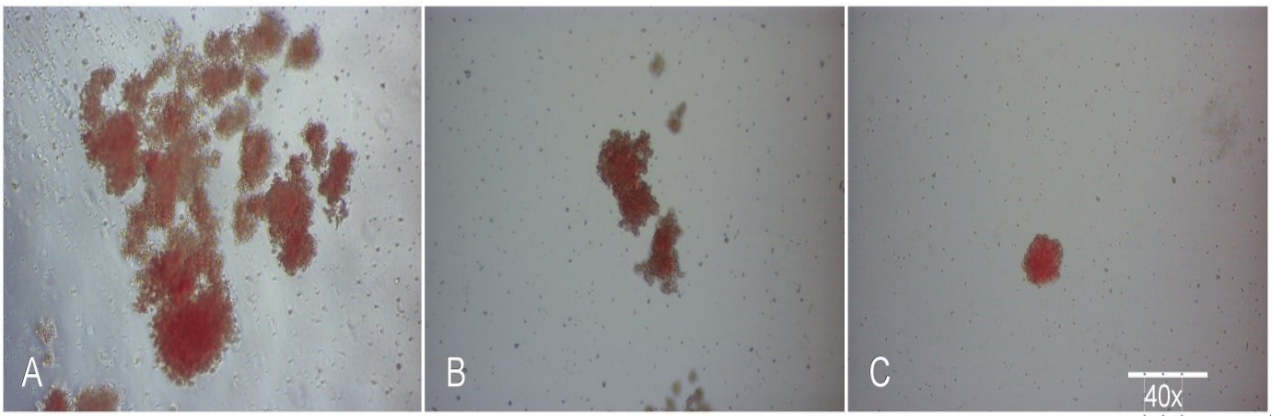


Figure 57: Typical colony sizes (40x) observed for BFU-E in untreated (A), 100 nM RUX (B) and 250 nM RUX (C). Colony size was markedly reduced in ruxolitinib treated cells.

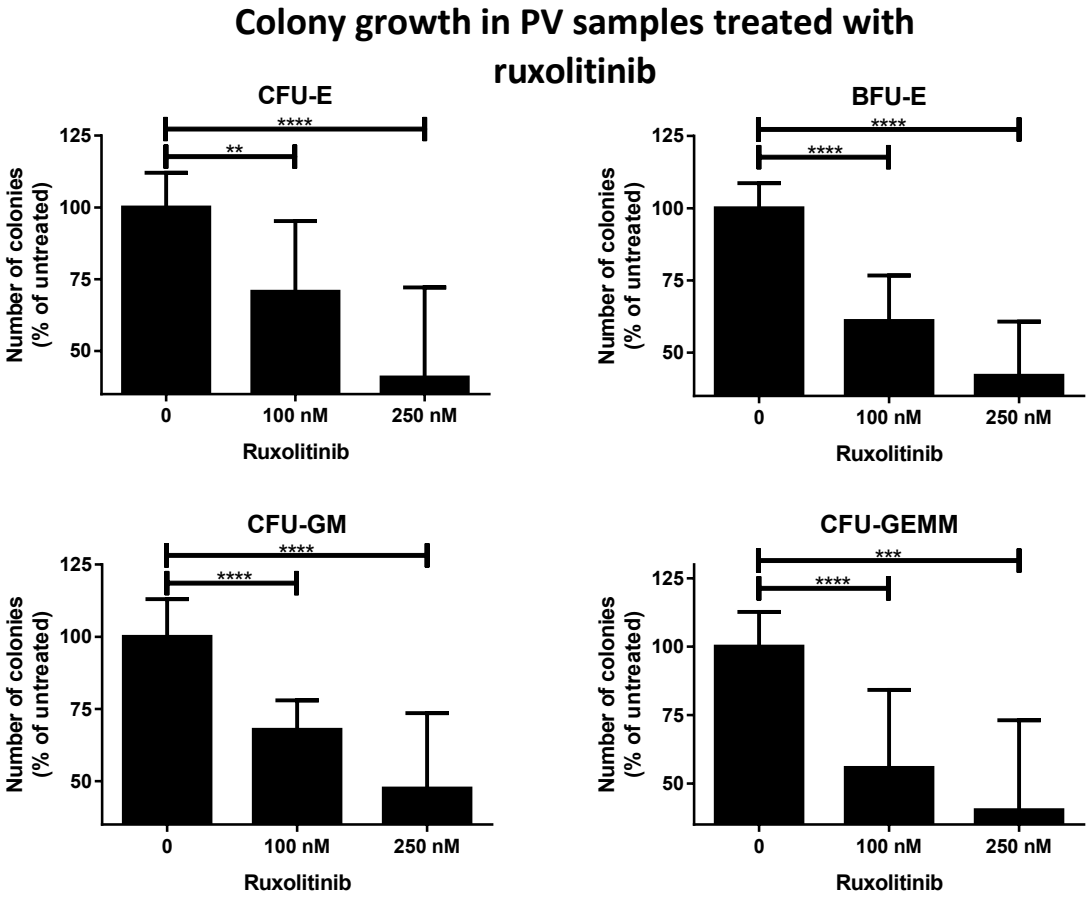


Figure 58: Colonies counted using the Stemcell Human CFU assay. A decrease in all colony types was seen in ruxolitinib treated PBMCs from PV patients (n = 4 independent patient samples ± SD).

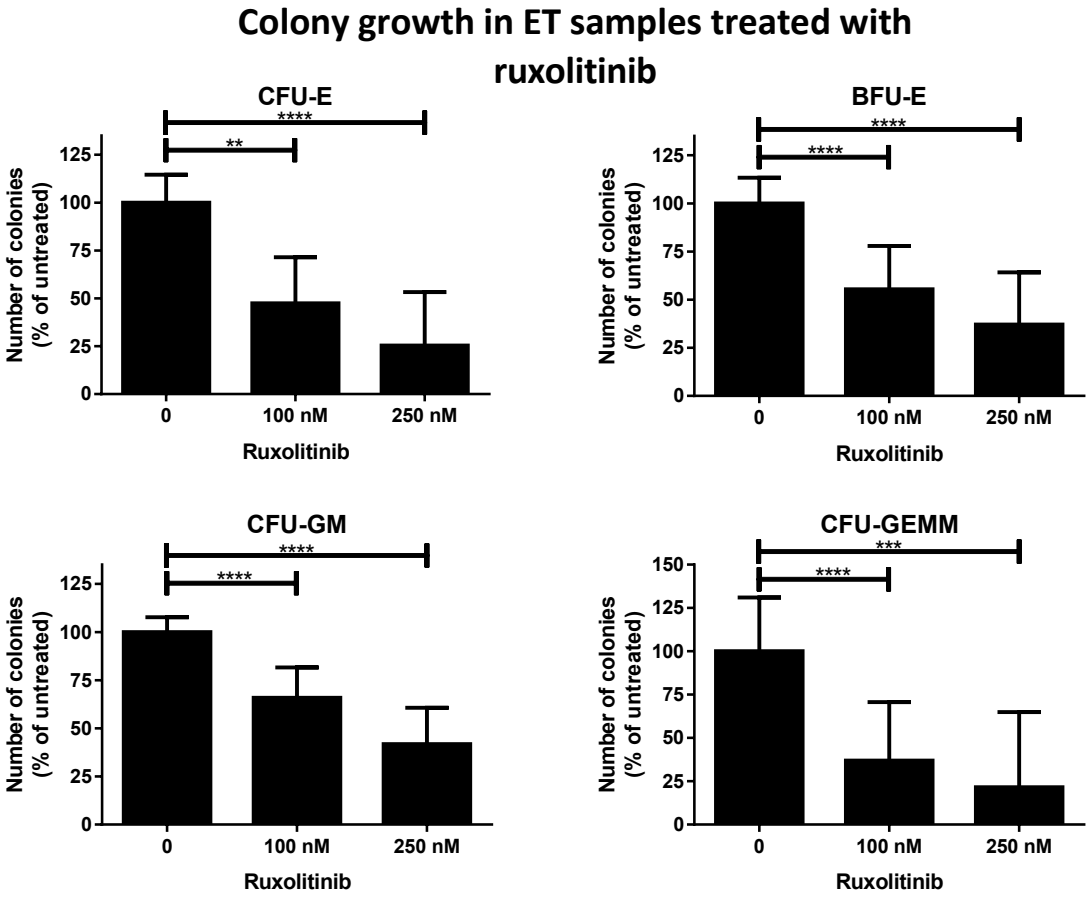


Figure 59: Colonies counted using the Stemcell Human CFU assay. A decrease in all colony types was seen in ruxolitinib treated PBMCs from ET patients (n = 4 independent patient samples ± SD).

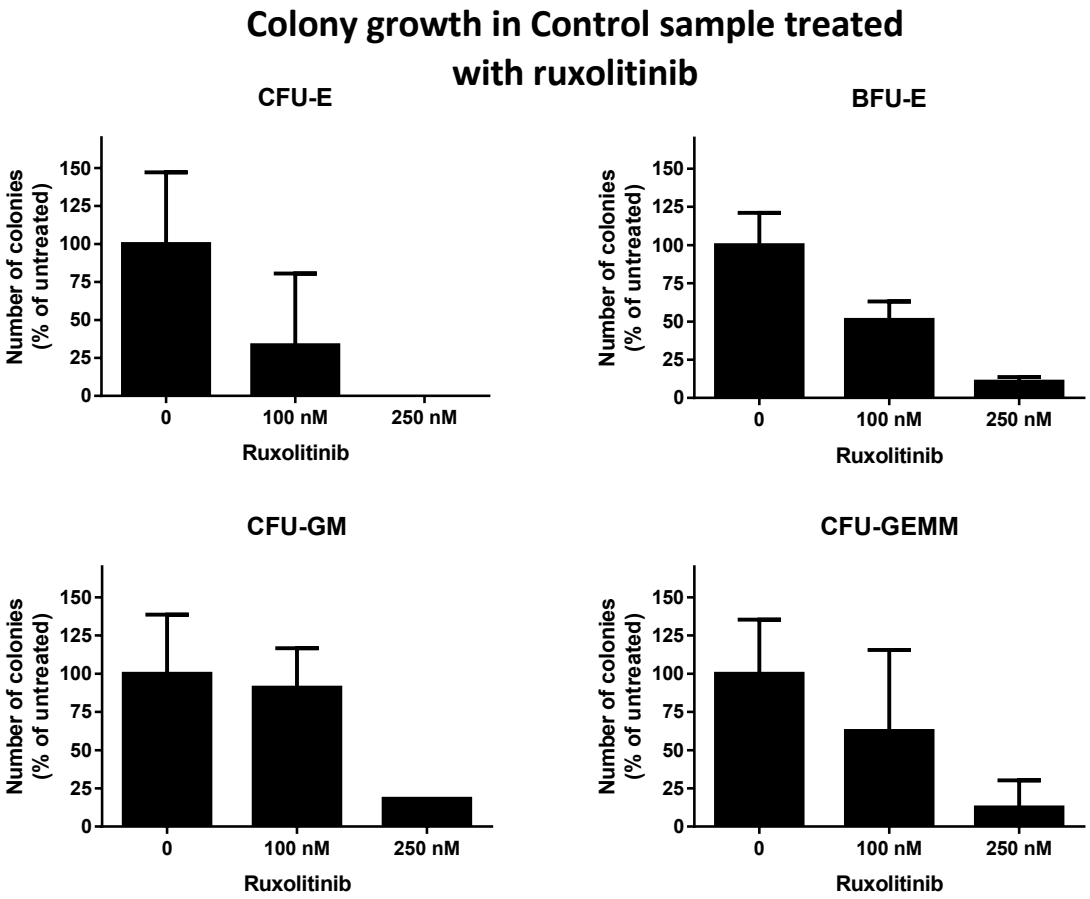


Figure 60: Colonies counted using the Stemcell Human CFU assay. A decrease in all colony types was seen in ruxolitinib treated PBMCs from a control patient.

5.4.5: Ruxolitinib treatment of SET2 cells results in the differential expression of 187 unique proteins

To determine the molecular effects of ruxolitinib inhibition on JAK2 signalling, high resolution quantitative mass spectrometry was employed on the MPN cell model SET2. An overlay of the total ion chromatograms for each instrument run is shown in Figure 61 for the fractionated samples. A representative total ion chromatogram is shown in Figure 62.

Relative quantification mass spectrometry was employed to identify pathways affected by ruxolitinib inhibition of JAK2. Its effects on 3113 proteins were quantified with high confidence and a false discovery rate <1%; indicating good coverage of the total cellular proteome. The labelling efficiency was > 98%, determined by comparing the total number of potential reactive sites (i.e., N-termini and lysine side chains) with the iTRAQ modification present, with two iTRAQ tags required for proteins to be quantified. Upon ruxolitinib treatment (1 μM), 187 total proteins were identified as being significantly differentially expressed, of which 26 were upregulated and 161 were downregulated (< 0.8 versus sample treated with vehicle control for at least two labels (113, 115, or 117) with a *p*-value < 0.05) (Table 13 in Chapter 7: Appendices).

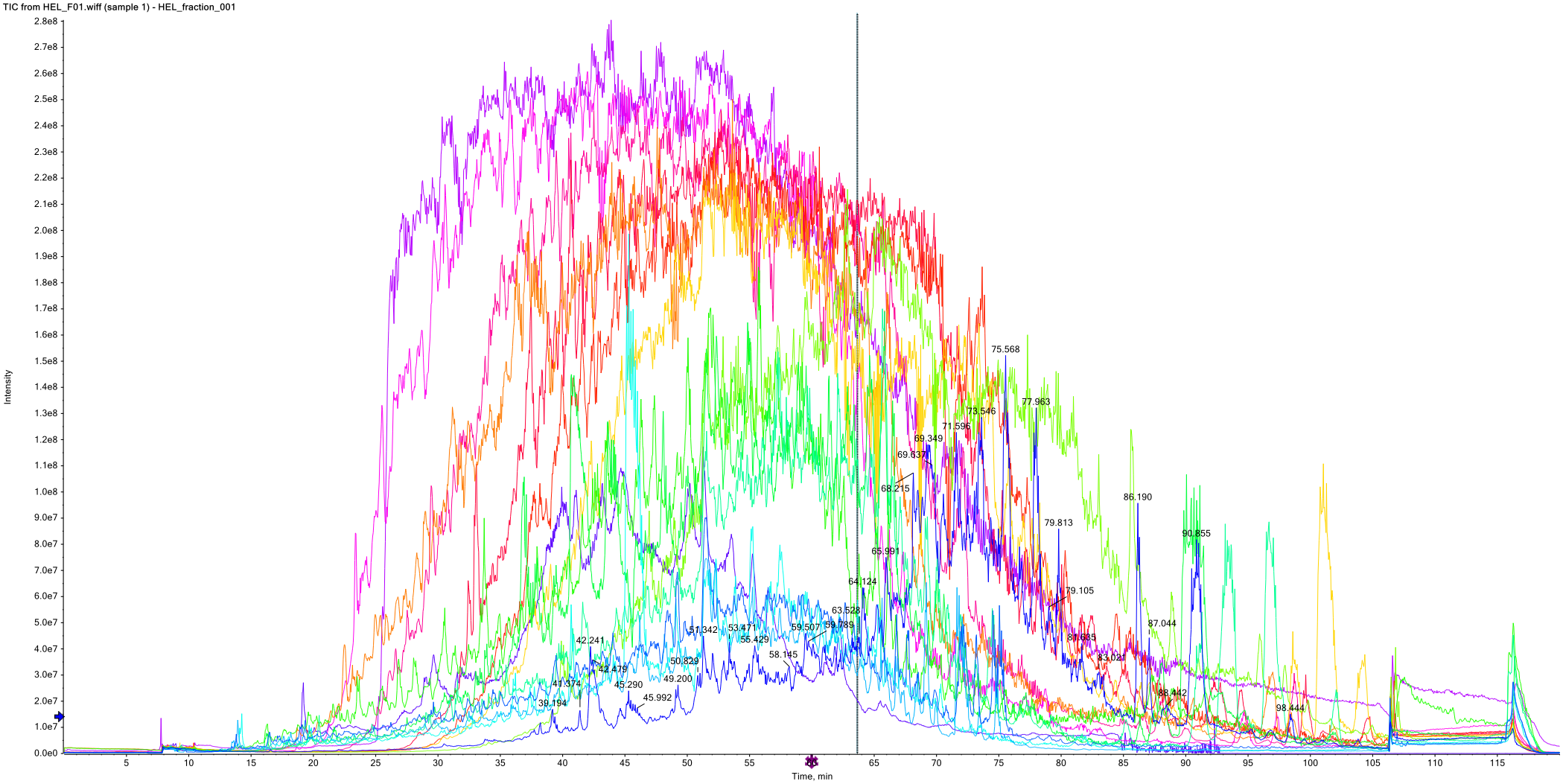


Figure 61: Overlay of total ion chromatograms for SET2 ± 1 µM RUX replicates labelled with iTRAQ reagent. Retention time is shown on the x-axis, (0 – 120 minutes). Intensity of peaks is displayed on the y-axis

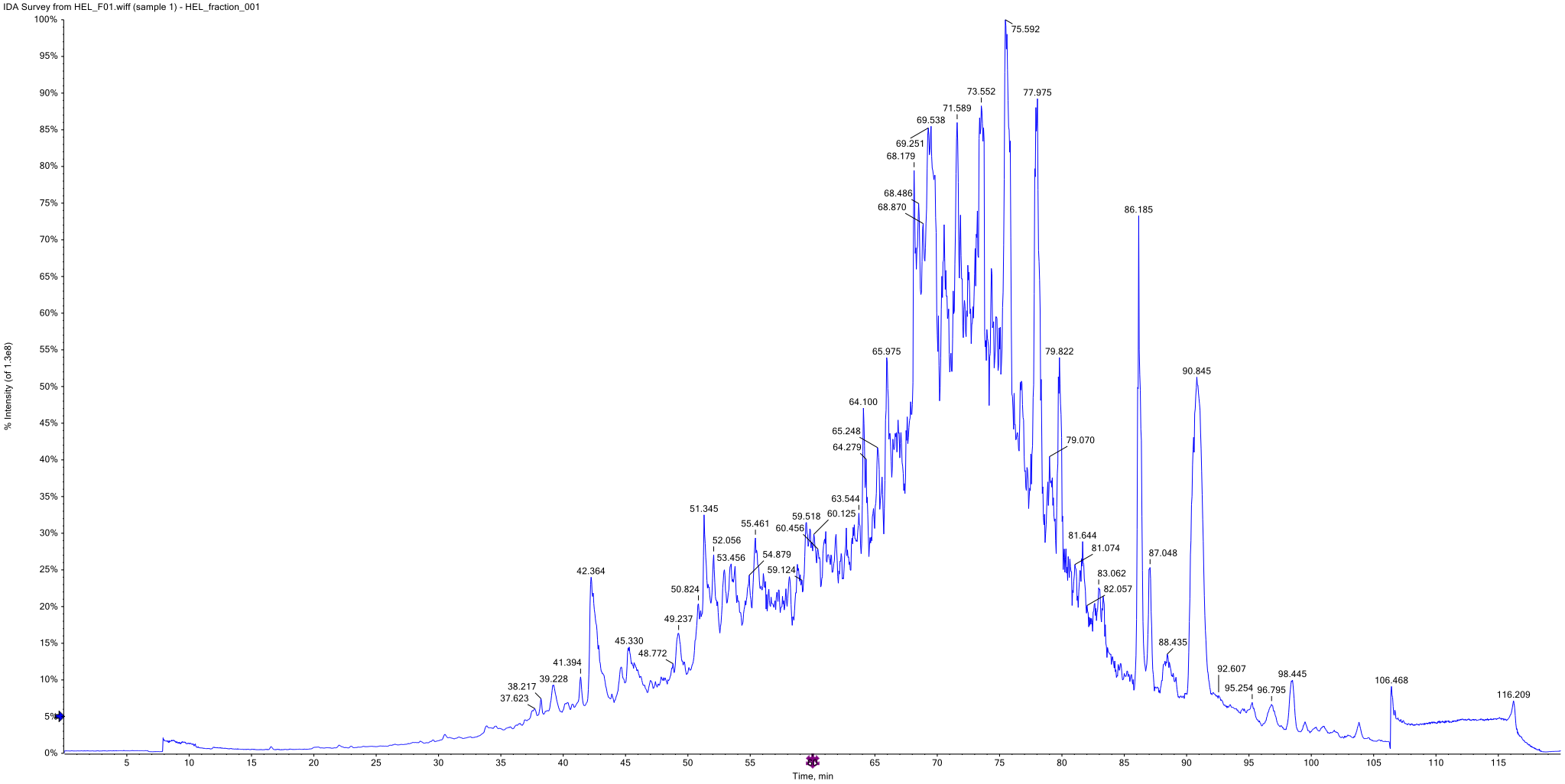


Figure 62: Representative TIC from one iTRAQ run from overlay shown in Figure 61. Time in minutes is on the x-axis (0 – 120) and % intensity is on the y-axis.

5.4.5.1: Functional characterisation of downregulated proteins in SET-2 upon ruxolitinib treatment.

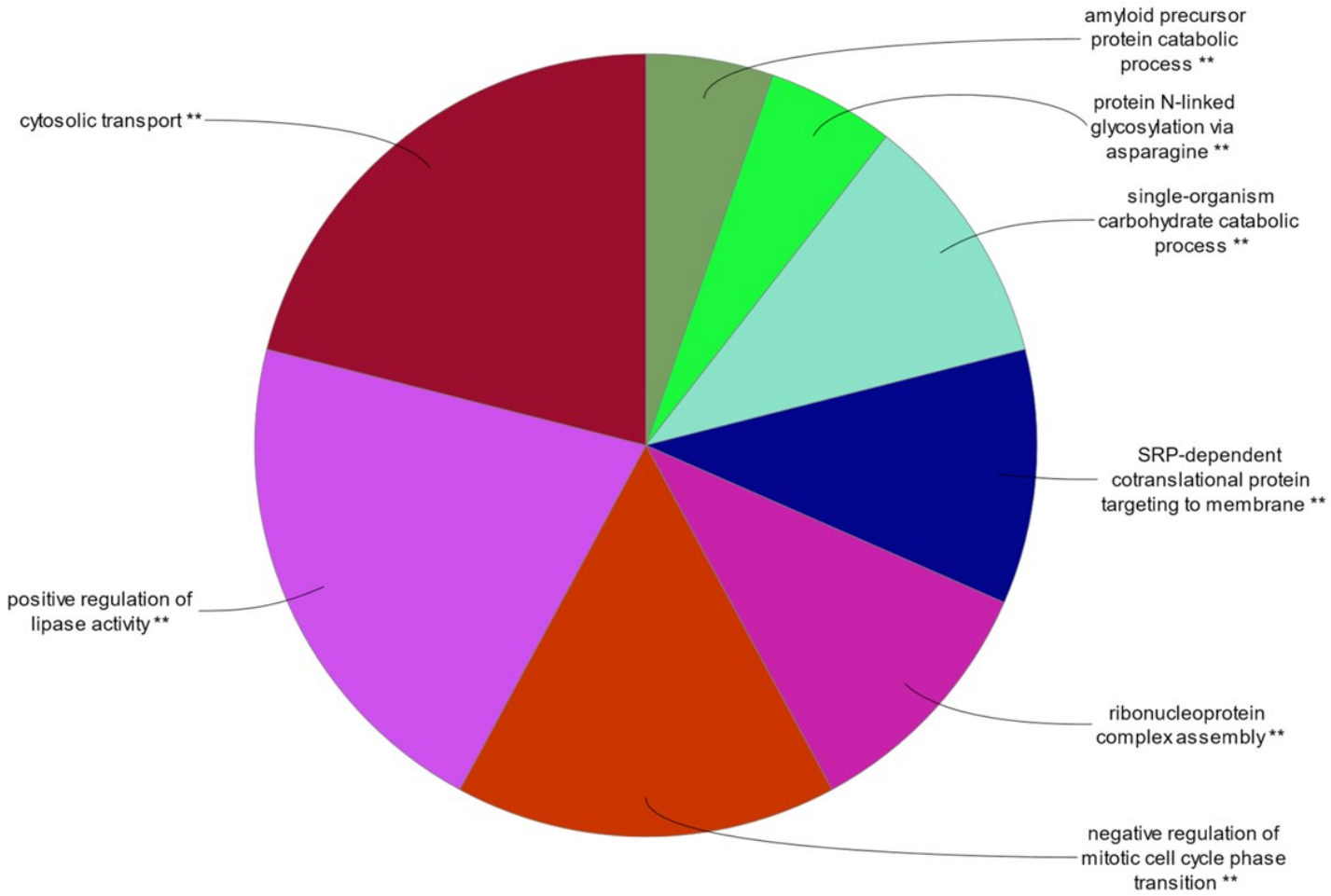


Figure 63: Pie chart of overrepresented gene ontology (GO) biological process terms for downregulated proteins (< 0.8) in SET2 cells treated with 1 µM ruxolitinib measured by mass spectroscopy.

The molecular functions, localisation and expression of individual proteins are well characterised and curated in databases such as Uniprot (UniProt, 2015). However, proteins rarely act alone in modulating cell and biological processes (De Las Rivas and Fontanillo, 2010). With the large number of differentially expressed proteins from iTRAQ experiments (Table 13 in Chapter 7: Appendices), it is necessary to examine the networks these proteins belong to as well as how they affect other processes, directly and indirectly. To determine how these proteins interact with each other and other molecular pathways, a program called STRING was utilised (Szklarczyk *et al.*, 2015). STRING applies weighting to protein-protein interactions dependent on known experimental data, as well as computationally predicted interactions. STRING analysis was used to map protein-protein interactions from the differentially expressed data set (Figure 64). Minimum interaction score was set at > 0.700 for high confidence. A maximum of five node separation was used to calculate indirect associations.

The analysis highlighted eight interacting proteins with functional enrichment in ribosome pathway (false discovery rate: 0.000195). These were connected to a group of aminoacyl-tRNA synthetase (ARS) proteins, IARS, QARS, and DARS. ARSs are involved in the initial stages of protein synthesis, they catalyse esterification reactions ligating transfer RNAs with their cognate amino acids (Park *et al.*, 2008). In addition to their canonical roles in transcription, these proteins have also been reported to control functions such as tumorigenesis, angiogenesis, and inflammation (Kim *et al.*, 2011). Mammallian ARS proteins form a large multi-protein complex (along with NS1 associated protein, ribosomal protein L13a, and GAPDH) in monocytes exposed to interferon-γ, called the gamma-interferon induced inhibitor of translation (GAIT) (Ribas de Pouplana and Geslain, 2008). The GAIT complex binds to a RNA motif in the 3’ untranslated region of target inflammatory mRNAs, thereby preventing translation by blocking the interaction between eukaryotic translation initiation factor 4G1 (eIF4G) and eIF3 (Carpenter *et al.*, 2014).

The group of eight ribosomal proteins are also linked to guanine nucleotide-binding protein, β2-like 1 (GNB2L1), also known as receptor for activated C kinase 1 (RACK1) (Adams *et al.*, 2011). Originally

identified as a scaffolding binding protein for protein kinase C (Adams *et al.*, 2011), it is now also known to interact with non-receptor tyrosine kinases, such as Src kinases (Mamidipudi *et al.*, 2007a, Mamidipudi *et al.*, 2007b, Chang *et al.*, 2002b), as well as receptor tyrosine kinases, insulin-like growth receptor factor 1 (IGF-R1) (Kiely *et al.*, 2002), and type I interferon receptor (Usacheva *et al.*, 2001). RACK1 mediates STAT3 recruitment and activation through IGF-R1 and insulin receptor (Zhang *et al.*, 2006). RACK1 has also been found to interact with STAT1, through the type I interferon receptor (Usacheva *et al.*, 2001, Kubota *et al.*, 2002).

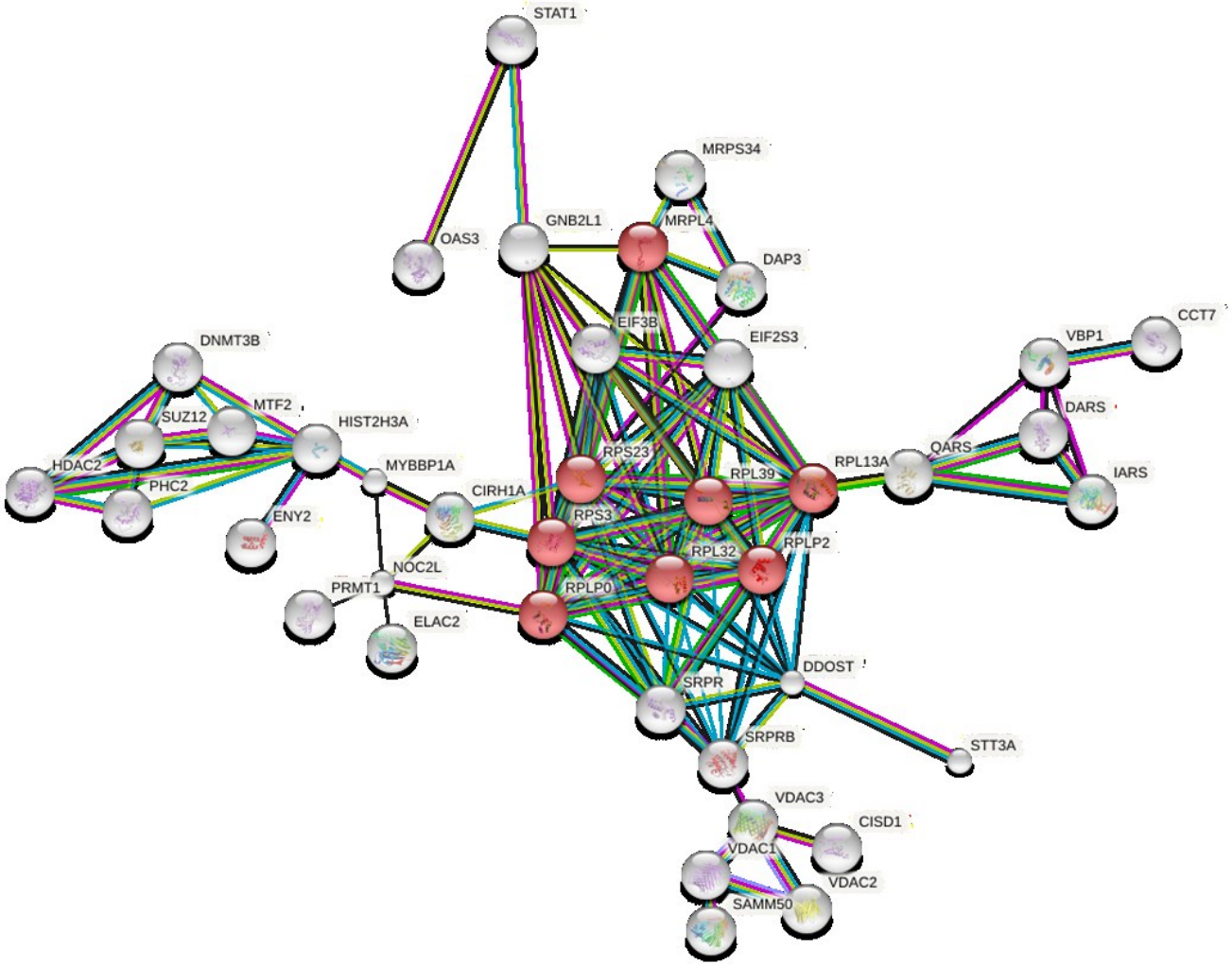


Figure 64: STRING analysis of protein-protein interactions identified from SET2 cells treated with 1 µM ruxolitinib. Minimum required interaction score was > 0.700 (high confidence). Proteins associated with ribosome pathway (in red) were significantly enriched.

5.4.6: STAT1 protein levels, but not STAT3/5, decrease in response to ruxolitinib treatment in *JAK2*^{V617F} cell lines

Following on from the global proteomic analysis, where ruxolitinib was shown to affect levels of proteins involved in antigen processing and presentation, it was decided to examine expression of STAT signalling proteins. Relative protein quantification using iTRAQ labelled peptide digests of SET2 cells treated with ruxolitinib showed that only STAT1 expression was significantly reduced ($p < 0.05$) compared to a control protein, GAPDH (Figure 65). There was a 2.5-fold reduction in STAT1 compared to GAPDH (Table 12). No significant ($p < 0.05$) change was observed for STAT3 ($p = 0.6867$) and STAT5A ($p = 0.6574$) (Table 12 and Figure 65).

Table 12: Relative protein quantification for STAT proteins measured by mass spectroscopy

Name	Uniprot ID	Unused protein score	113 FC	115 FC	117 FC	119 FC	**Avg FC**
GAPDH	P04406	42.26	0.8790	0.9290	1.0000	1.0965	**0.9360**
STAT1	P42224	22.01	0.5346	0.4365	0.2270	0.0673	**0.3994**
STAT3	P40763	11.82	1.0186	0.9120	1.4191	0.0667	**1.1166**
STAT5A	K7EK35	19.88	1.0000	1.0568	1.0093	0.9376	**1.0220**

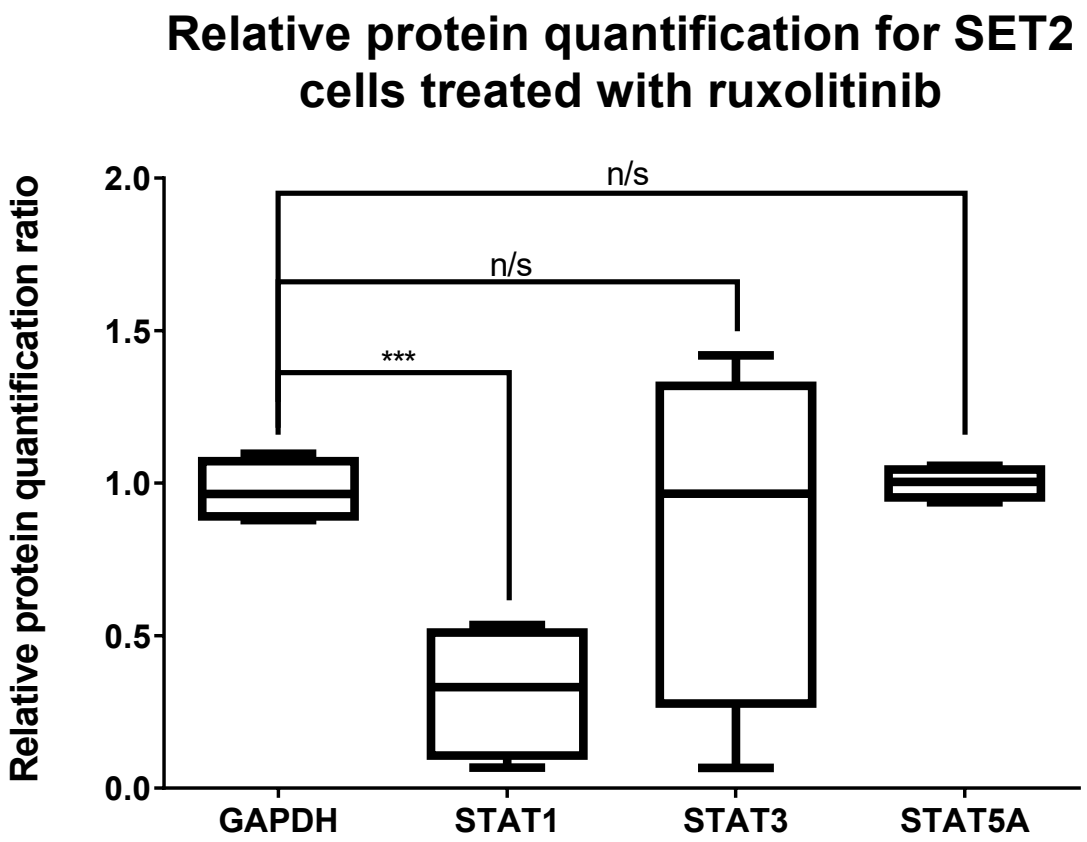


Figure 65: STAT protein levels measured by iTRAQ using LC-MS/MS. Treated samples (1 µM RUX) labelled with 113, 115, 117, and 119 isobaric tags. Ratios between all tags compared to 121 labelled control sample. T-test between GAPDH (control protein) and STAT protein ratios, STAT1: $p < 0.05$, STAT3: $p > 0.05$, STAT5A: $p > 0.05$.

In SET2, exposure to ruxolitinib resulted in dephoshorylation of STAT1, but also a reduction in the total level of STAT1 (Figure 66). This was also confirmed in the HEL cell line (Figure 67), which showed that as well as protein dephosphorylation in STAT1/3/5, there was a decrease in total STAT1 after 48 hours treatment. The reduction in both total and phosphorylated STAT1 was dose-dependent, with higher concentrations of ruxolitinib eliciting a greater response (Figure 66 and Figure 67). These western blot results reflect what was found in the mass spectroscopy analysis of STAT protein expression (Figure 65).

While phosphorylated STAT1 and STAT5 levels were decreased after 15 minutes exposure to ruxolitinib, total protein levels were unaffected up to 6 hours post-treatment in both HEL and SET2 cell lines (Figure 68).

Additionally, examination of STAT1 protein levels from the PBMCs of MPN patients treated overnight with ruxolitinib showed some interesting results. All patients showed a decrease in overall STAT1 protein, but this was insignificant for the two ET patients (Figure 70 and Figure 71) tested ($p > 0.05$). However, the sole PV patient (Figure 69) did have a significantly lower amount of STAT1 ($p < 0.05$) in PBMCs exposed to ruxolitinib. Further samples would be required to confirm these findings but initial results may suggest a link between response to ruxolitinib and *JAK2*^{V617F} allele burden, which is typically higher in PV patients (Passamonti and Rumi, 2009).

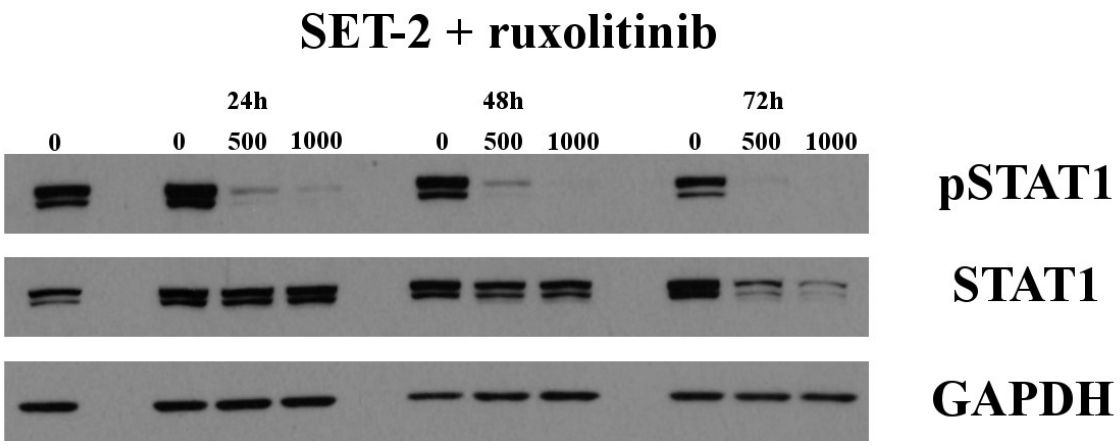


Figure 66: Protein expression in SET2 cells treated with ruxolitinib (0, 500, or 1000 nM) for 72 hours. Levels of phosphorylated STAT proteins decreased along with total STAT1 expression. Total STAT1 protein was reduced after 48 hours with 500 and 1000 nM.

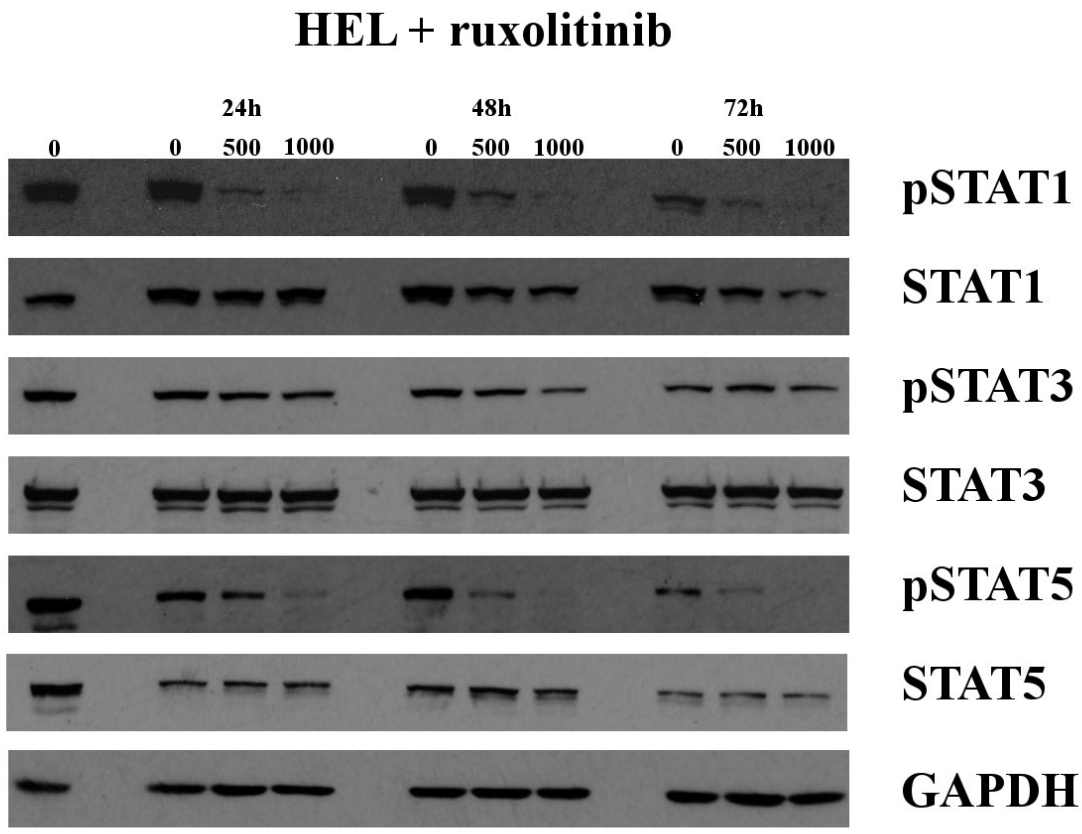


Figure 67: Protein expression in HEL cells treated with ruxolitinib (0, 500, or 1000 nM) for 72 hours. Levels of phosphorylated STAT proteins decreased along with total STAT1 expression. Total STAT1 protein was reduced after 48 hours with 500 and 1000 nM, confirming the findings with SET2 in Figure 66. There was no decrease in the level of total STAT3 or STAT5.

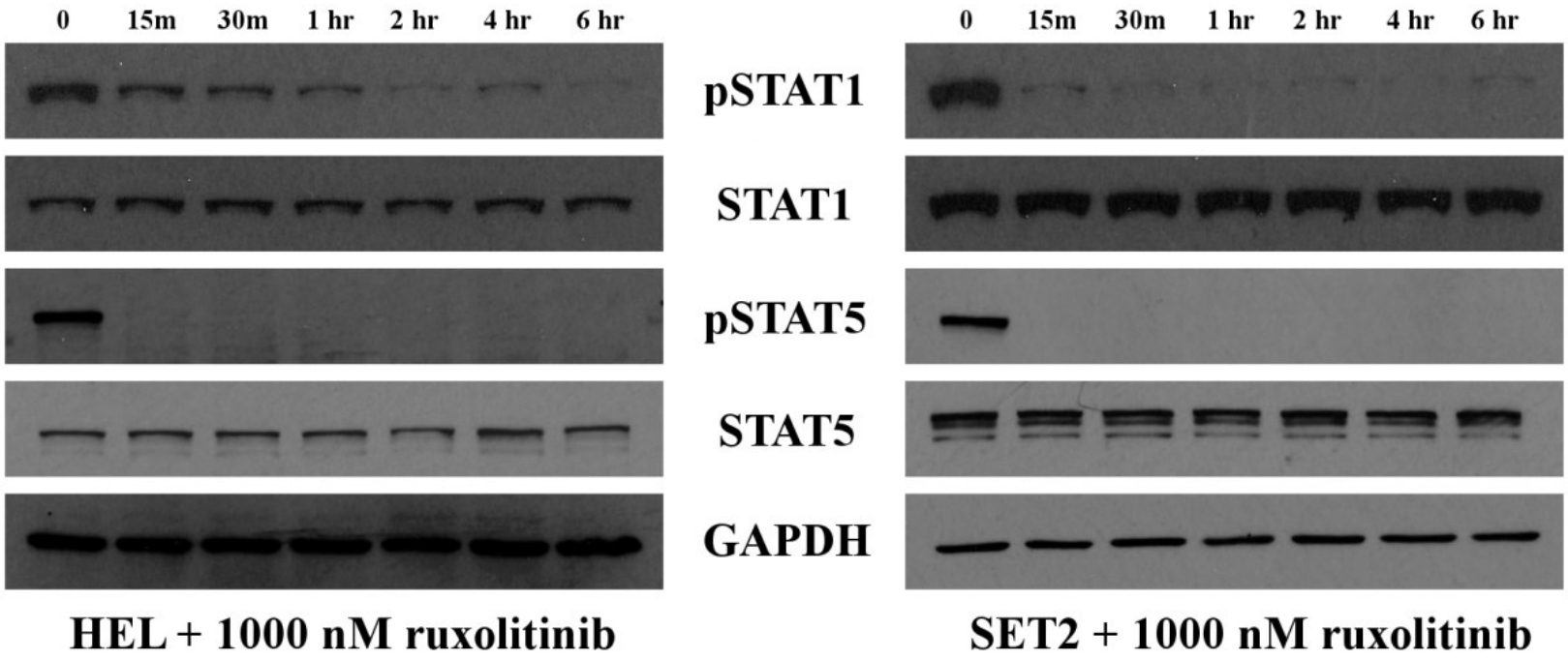


Figure 68: Cell lines (HEL and SET2) treated with ruxolitinib for 6 hours. Decrease in phosphorylated STAT1 and STAT5 was observed for both cell lines after 15 minutes treatment.

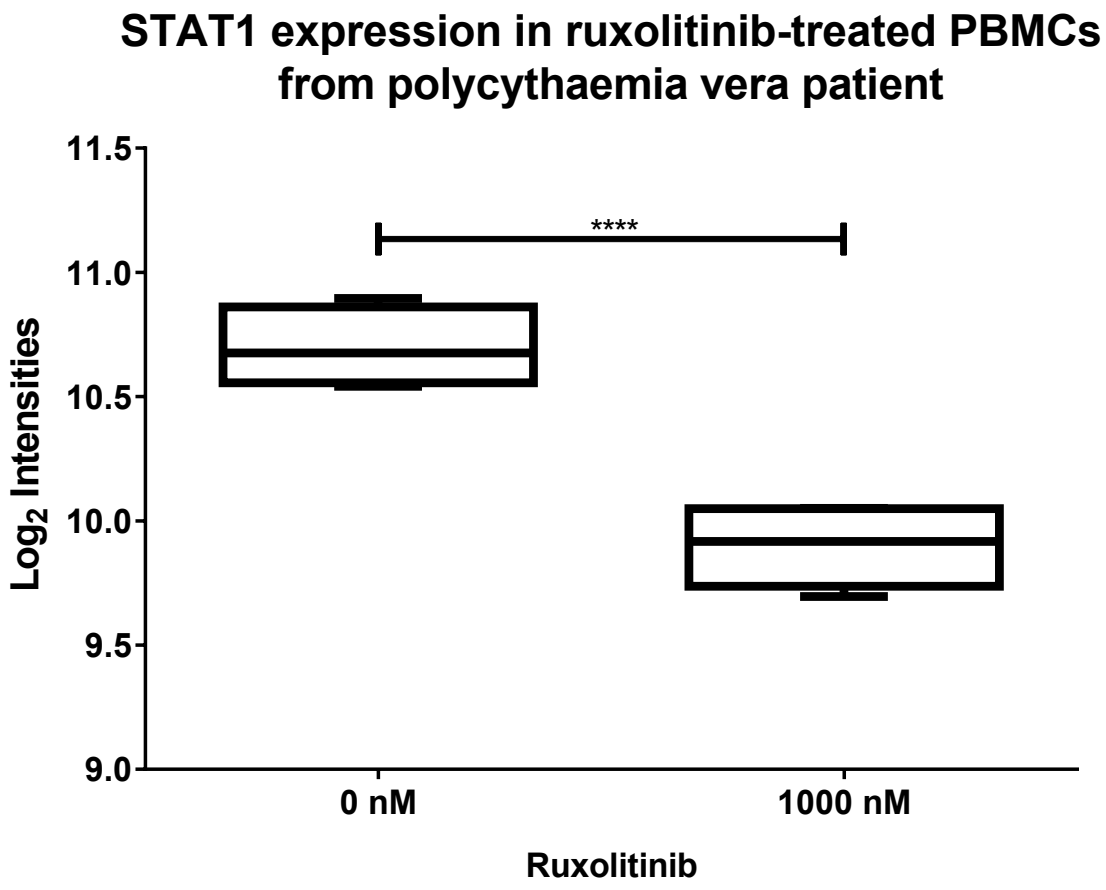


Figure 69: Relative protein levels of STAT1 measured by LC-MS/MS for PBMCs extracted from a polycythaemia vera patient blood sample in at least four mass spectroscopy runs (t-test; $p < 0.05$). PBMCs were treated overnight with ruxolitinib before extraction for mass spectroscopy.

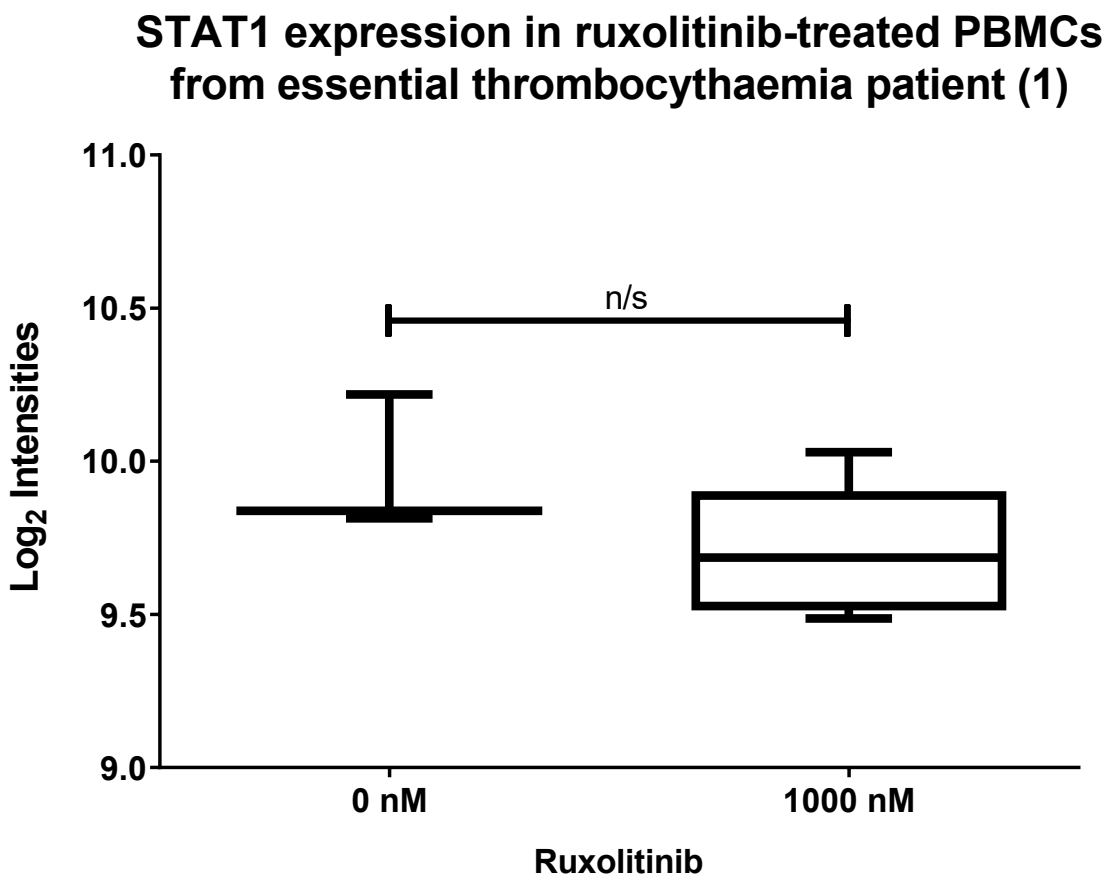


Figure 70: Relative protein levels of STAT1 measured by LC-MS/MS for PBMCs extracted from an essential thrombocythaemia patient blood sample in four mass spectroscopy runs. PBMCs were treated overnight with ruxolitinib before extraction for mass spectroscopy (t-test; $p > 0.05$).

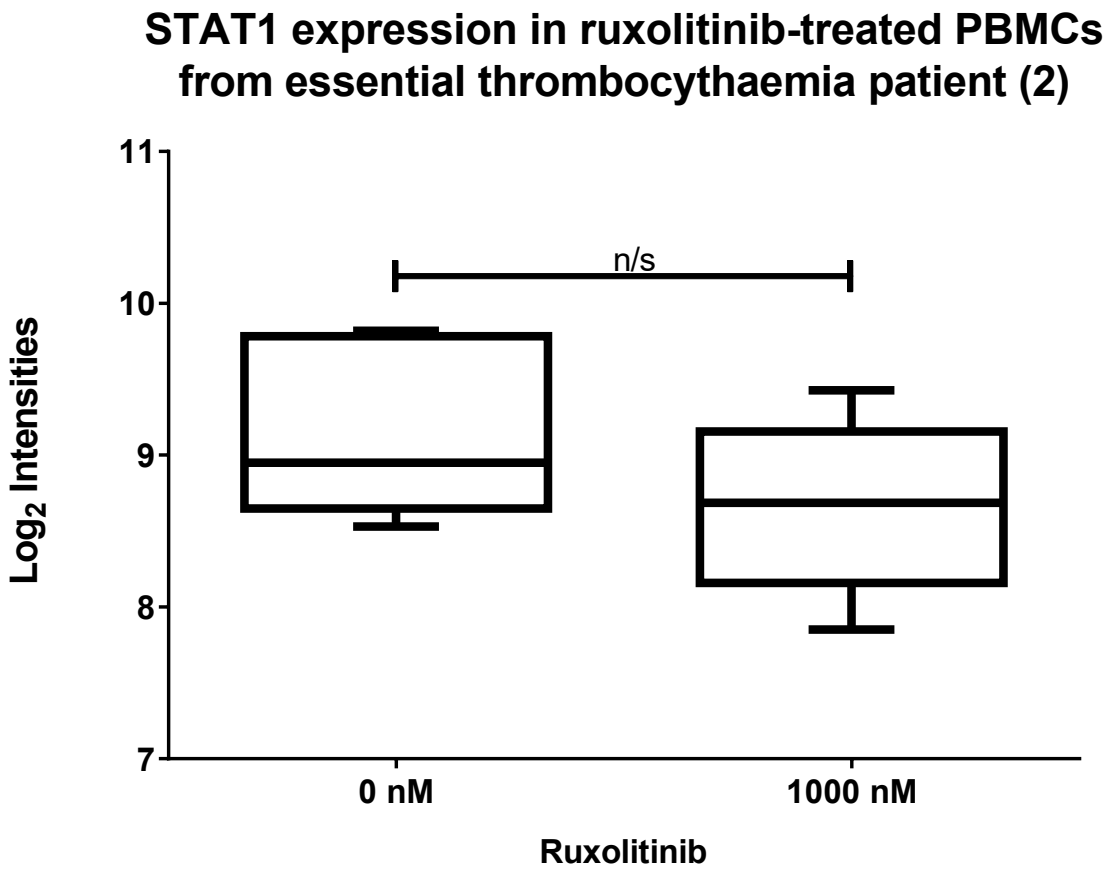


Figure 71: Relative protein levels of STAT1 measured by LC-MS/MS for PBMCs extracted from an essential thrombocythaemia patient blood sample. PBMCs were treated overnight with ruxolitinib before extraction for mass spectroscopy. Four mass spectroscopy runs carried out (t-test; $p > 0.05$).

5.4.7: *STAT1* mRNA is reduced in *JAK2*^{V617F}, but not *JAK2*^{WT} cell lines

STAT1 total protein expression was shown to be reduced in Figure 65 - Figure **67**. To determine whether STAT1 downregulation occurred during translation or transcription, the levels of *STAT1* mRNA were measured in three cell lines using quantitative PCR upon RUX treatment. *STAT1* was decreased in both HEL (0.67 x control) and SET2 (0.37 x control) cell lines, but slightly increased in the K562 cell line (Figure 72). The greater knockdown of *STAT1* in the SET2 cell line compared to HEL, corresponds to its increased sensitivity to ruxolitinib treatment, shown in Figure 47 and Figure 48. Total STAT1 protein reduction in response to ruxolitinib in these cell lines is likely to be because of inhibition of *STAT1* transcription.

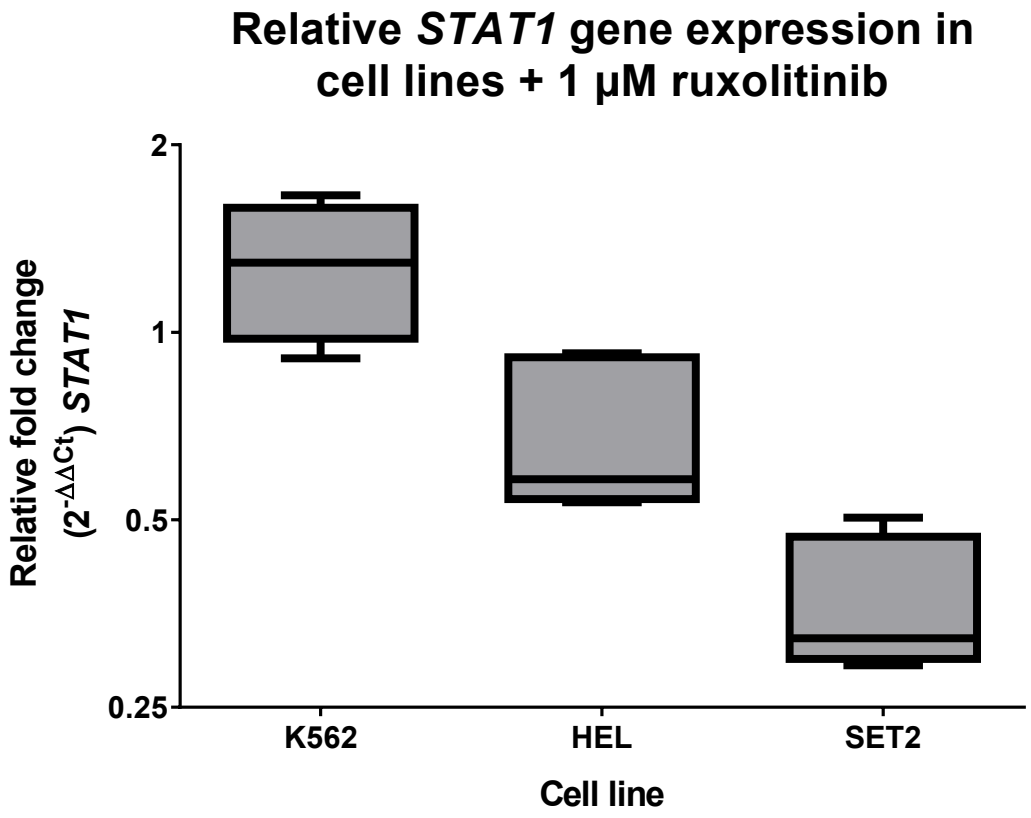


Figure 72: *STAT1* gene expression in K562 (*JAK2*^{WT}), HEL and SET2 (*JAK2*^{V617F}) treated with 1 µM ruxolitinib for 48 hours (n = 3 ±SD). Gene expression normalised using the housekeeping gene, *GAPDH* and a vehicle control treated sample. *STAT1* levels were reduced in both *JAK2*^{V617F} cell lines compared to samples treated with vehicle control.

5.4.8: *SOCS3* is downregulated in SET2 cells treated with ruxolitinib

Next, the expression levels of cytokine-inducible negative regulators of cytokine signalling (SOCS proteins) were measured. Expression of *SOCS1* was increased 1.5-fold whereas there was a two-fold reduction in *SOCS3* (Figure 73). Both these proteins reduce cytokine signalling in a classical negative feedback loop, where their expression is induced along with the target cytokine or downstream target (Krebs and Hilton, 2001). The downregulation of *SOCS3* is consistent with the decrease in STAT1 activity in response to ruxolitinib (Figure 67). The increase in *SOCS1* may be as a result of paradoxical JAK2 phosphorylation by ruxolitinib. Differential response to type I or type II interferon signalling may also be responsible for the differences between *SOCS1* and *SOCS3* expression.

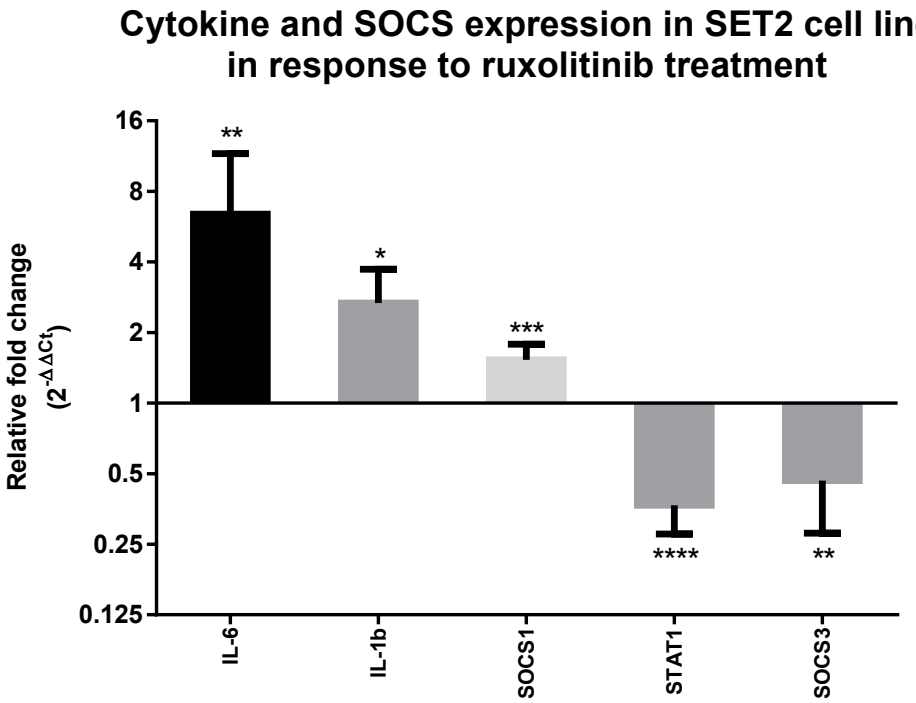


Figure 73: Gene expression of inflammatory cytokines (*IL-6, IL-1b*) and SOCS proteins relative to *GAPDH* reference gene and vehicle control treated sample. Levels of *IL-1b* and its downstream target, *IL-6*, were upregulated ($p < 0.05$) in response to ruxolitinib treatment of SET2 cells. There was an increase in the level of *SOCS1* and a decrease in *SOCS3* expression ($p < 0.05$) (n = 3 ± SD).

5.4.9: Ruxolitinib is a more potent inhibitor of *STAT1* transcription than fludarabine

The effect of fludarabine, a chemotherapy drug used in the treatment of CLL and a STAT1 inhibitor at RNA and protein level, was examined in the SET2 cell line (Figure 74). 50% inhibition of proliferation in the SET2 cell line was achieved using 5.5 µM of the drug over 72 hours (Figure 74). Both ruxolitinib and fludarabine treatment in SET2 cells resulted in a downregulation of STAT1 (Figure 75). Ruxolitinib treatment in SET2 at 1 µM resulted in a two-fold reduction of *STAT1*. The reduction induced by fludarabine was more modest (0.7x control), although this was at a level equivalent to the IC_{50} calculated in Figure 74.

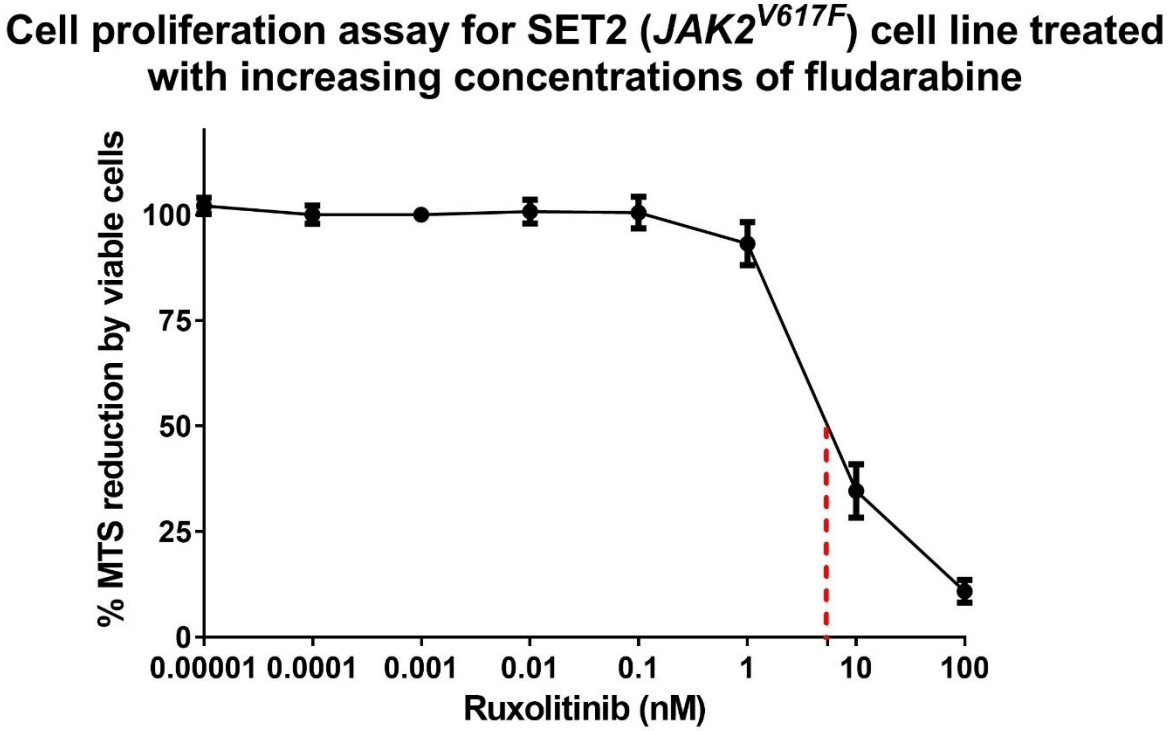


Figure 74: Cellular proliferation assay of the STAT1 inhibitor, fludarabine, on the *JAK2*^{V617F} cell line SET2. Proliferation was reduced in response to increasing concentrations of fludarabine. IC_{50} value determined using GraphPad Prism software: 5.5 µM (n = 3 ± SD).

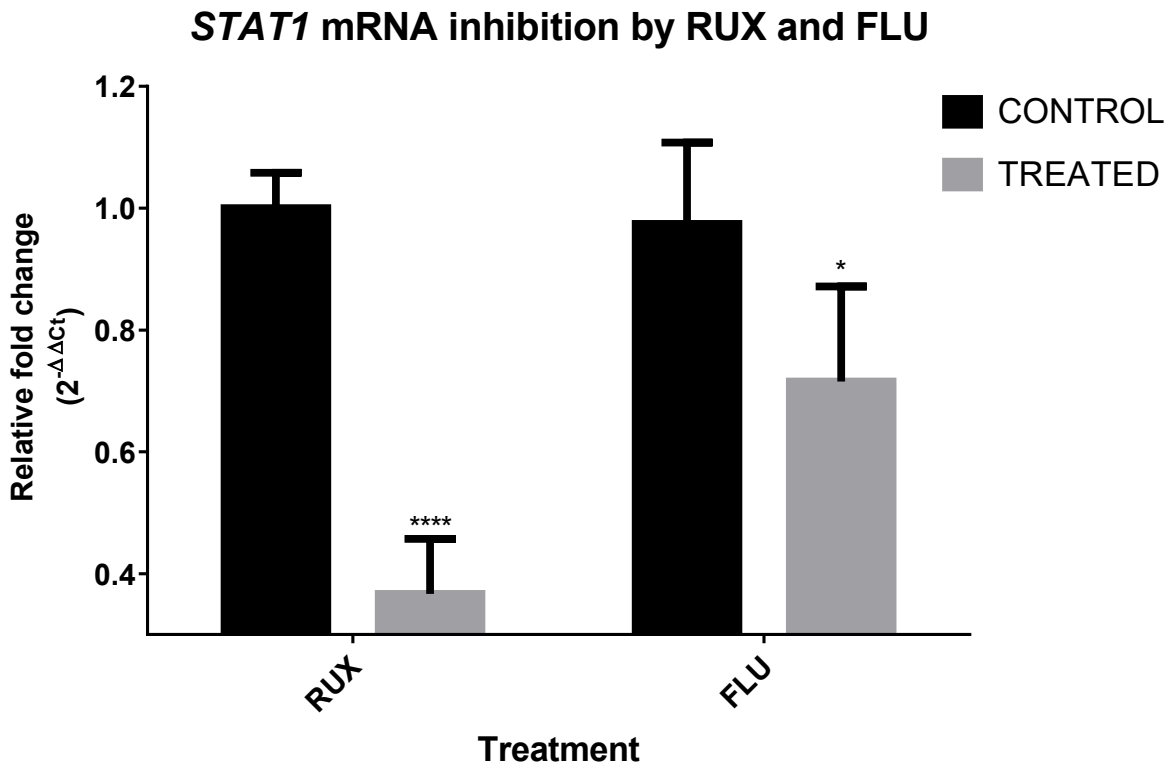


Figure 75: *STAT1* mRNA expression in response to ruxolitinib or fludarabine treatment. *STAT1* is downregulated by ruxolitinib (1 µM) and the STAT1 specific inhibitor, fludarabine (5.5 µM).

5.5: Discussion

Ruxolitinib, a JAK1/2 inhibitor, reduced proliferation on all *JAK2*^{V617F} cell lines (HEL, SET2 and UKE1) (Figure 47 - Figure 49), but not on *JAK2*^{WT} cells, K562 and HL60 (Figure 50 and Figure 51). At the concentrations tested, the inhibition appeared specific to cells bearing the *JAK2*^{V617F} mutation, despite the mechanism of ruxolitinib being targeted against all JAK2 activity, regardless of mutational status. Other proliferation assays using WST1 reagent, have shown similar IC_{50} values for HEL (790 nM) and SET2 (160 nM) when treated with ruxolitinib (Bogani *et al.*, 2013) as these results have shown (565 and 132 nM respectively). As reported by Quintás-Cardama *et al.* (2010), cells bearing the *JAK2*^{V617F} appear to selectively undergo apoptosis when treated with ruxolitinib, although clinically the drug is equally effective in patients with or without the *JAK2*^{V617F} mutation. The exact cause for this is not fully understood and given that non-*JAK2*^{V617F} patients do not display constitutive JAK/STAT activation (Schwemmers *et al.*, 2007), it would appear that ruxolitinib may have direct effects downstream from this signalling pathway, common to both *JAK2*^{V617F} and non-mutated *JAK2*.

Ruxolitinib did not affect the distribution of K562 cells in each phase of the cell cycle (Figure 52 and Figure 53). In K562 cells, STAT5 is constitutively activated through tyrosine phosphorylation by BCL-ABL (de Groot *et al.*, 1999, Hantschel *et al.*, 2012), thereby negating the effect of targeting JAK2 kinase activity upstream. After 48 hours, there was a significant increase ($p < 0.05$) in HEL cells in the G0/G1 phase and a decrease in cells in S phase indicating that this drug may cause a G0/G1 block in the cell cycle (Figure 54 and Figure 55). Similar results have also been demonstrated in Ba/F3-EPOR VF cells containing the *JAK2*^{V617F} mutation (Bogani *et al.*, 2013). A key regulator of G1/S progression CDC25A has been reported to be a target for constitutive *JAK2*^{V617F} activity and is overexpressed in murine and human cells (including HEL and SET2) bearing the *JAK2*^{V617F} mutation (Gautier *et al.*, 2012). JAK2 has also been shown to bind and phosphorylate p27(Kip1), a cyclin dependent kinase inhibitor (Jäkel *et al.*, 2011).

Ruxolitinib also inhibited the growth of haematopoietic progenitor cells in colony-forming unit assays from ET, PV, and control patients, indicating that it is effective against the mutant clone (Figure 33 - Figure 35). This effect has also been shown in mononuclear cells from both PV and ET (*JAK2*^{V617F} and *JAK2*^{WT}) (Barrio *et al.*, 2013), although notably this group found wild type *JAK2* in ET patients was more sensitive to ruxolitinib treatment. The sizes of BFU-E colonies were also significantly reduced on ruxolitinib treatment. BFU-E development and proliferation in these colonies is as a result of IL-3 stimulation of the JAK/STAT pathway (Callus and Mathey-Prevot, 1998, Dai *et al.*, 1991), which is inhibited on ruxolitinib treatment.

Both STAT3 and STAT5 phosphorylation was decreased in *JAK2*^{V617F} cells (Figure 56 and Figure 67) when treated with ruxolitinib. Constitutive STAT3 and STAT5 activation is a hallmark of MPNs with *JAK2*^{V617F} or exon 12 mutations. Levels of phosphorylation of the respective STAT proteins has been linked to disease phenotype (Teofili *et al.*, 2007). High levels of both phospho-STAT3 and phospho-STAT5 were found in bone marrow biopsies of PV patients, whereas high pSTAT3 and lower pSTAT5 was associated with an ET phenotype (Teofili *et al.*, 2007). Lower overall pSTAT3 and pSTAT5 was indicative of myelofibrosis (Teofili *et al.*, 2007). In the cell lines tested, SET2 was isolated from a patient with a previous history of ET, it was observed that STAT5 phosphorylation was weaker than pSTAT3. Ruxolitinib treatment was also able to completely knockdown pSTAT5, but only resulted in a partial reduction in pSTAT3. Similar STAT3/STAT5 phosphorylation patterns were seen in the HEL cell line, whose MPN status is unknown. It is not known why ruxolitinib did not fully inhibit STAT3 phosphorylation in the cell lines tested, since previous groups have shown this effect in the HEL (Quintás-Cardama *et al.*, 2010) and SET2 (Meyer *et al.*, 2015) cell lines. Insensitivity of these cells to type I JAK inhibitors is a possible explanation, although the IC_{50} value in cell-proliferation assays for the SET2 cells was 132 nM. This is similar to that found in naïve SET2 cells (70 nM) compared to 900 nM in ruxolitinib persistent SET2 (Meyer *et al.*, 2015). Both *JAK2*^{V617F} cell lines (HEL and SET2) showed hyper-phosphorylation of JAK2 when exposed to ruxolitinib (Figure 56). Ruxolitinib is a type I JAK inhibitor, and it targets the active kinase confirmation of JAK2 in an ATP-competitive manner. The

paradoxical phosphorylation of JAK2 and dephosphorylation of downstream STAT proteins has been observed by other groups (Andraos *et al.*, 2012, Meyer *et al.*, 2015). The mechanism for this is not fully understood, however experiments suggest that conformational changes induced by ruxolitinib may result in protection of the Tyr1007/1008 sites on JAK2 from phosphatases (Gorantla *et al.*, 2013). Type II JAK inhibitors, such as CHZ868, do not have this effect (Meyer *et al.*, 2015). As with type I inhibitors, type II JAK inhibitors bind to the ATP-binding site but also to an induced hydrophobic pocket. This has the effect of stabilising JAK2 in an inactive confirmation (Meyer *et al.*, 2015, Andraos *et al.*, 2012). Treatment with type II JAK inhibitors can also overcome persistence to type I inhibitors in SET2 cells. This occurs due to reactivation of the JAK/STAT pathway through heterodimerisation between JAK2 and JAK1/TYK2 (Koppikar *et al.*, 2012, Meyer *et al.*, 2015). CHZ868 can overcome persistence to type I JAK inhibitors in SET2 cells (Meyer *et al.*, 2015).

Global proteomic analysis using mass spectroscopy identified 162 proteins which were downregulated on ruxolitinib treatment (Table 13 in Chapter 7: Appendices). Using an analysis of GO terms relating to biological processes, it was found that there was an overrepresentation of terms relating to several processes involved in ribonucleoprotein complex assembly, protein glycosylation, and cytosolic transport (Figure 63 and Table 14).

STRING analysis of protein-protein interactions showed an enhancement of differentially regulated proteins associated with the ribosome pathway. These were separated by a single node to STAT1. STAT1 plays a key role in signal transduction from IFN-γ signalling via its membrane-bound receptor. IFN-γ is important in modulating cellular immunity to intracellular pathogens, inflammation, macrophage recruitment, and tumour suppression (Hu *et al.*, 2009). Activation of STAT1 is responsible for the transcription of a large number of downstream IFN-γ target genes (Krause *et al.*, 2006). It has also been shown in *GATA1* knockdown mouse models that STAT1 production is impaired along with impaired terminal megakaryopoiesis and this can be part rescued through ectopic expression of *STAT1* or its target interferon receptor factor 1 (*IRF1*) (Huang *et al.*, 2007). In addition, loss of *STAT1*

promotes erythropoiesis over megakaryopoiesis in *JAK2*^{V617F} mouse models (Duek *et al.*, 2014). *STAT1* and *JAK2* expression are downregulated in *GATA1* knockdown murine megakaryocytes (Muntean and Crispino, 2005). Ectopic expression of *STAT1* or its target effector, *IRF1*, can rescue megakaryopoiesis defects in these GATA1 deficient mice (Huang *et al.*, 2007).

Relative protein quantification between STAT proteins (1, 3, and 5A) and a control gene (GAPDH) showed that only STAT1 was significantly downregulated, 2.5-fold lower than GAPDH, in response to ruxolitinib (Table 12 and Figure 65). In western blot experiments using cells treated over 6 hours, no change in total STAT for either STAT1 or STAT5 was observed. Phosphorylated levels of these proteins were reduced after 15 minutes. Longer experiments over 72 hours did match the results observed in mass spectroscopy experiments. Levels of total STAT1 reduced after 48 hours, while STAT3 and STAT5 remained unchanged. Differences in *STAT* expression, between STAT1 (normally involved in the upregulation of apoptotic genes) and STAT5, is one potential mechanism proposed to explain how distinct phenotypes can arise from the same somatic mutation (Chen *et al.*, 2010). This may also have implications in the design of drugs that target JAK2 and by association STAT phosphorylation and signalling.

STAT1 and STAT3 have contrasting roles in controlling cell cycle progression and tumourigenesis. STAT1 negatively regulates the cell cycle through expression of cyclin-dependent kinase (CDK) inhibitors, including p21$^{waf/cip1}$ and p27^{Kip1}, as well as the G1/S phase blocker, KLF4 (Avalle *et al.*, 2012). STAT3 is responsible for activating CDKs, through increased expression of cyclin D2 as well as downregulating p21, a CDK inhibitor (Thomas *et al.*, 2015). In multiple myeloma cells, STAT1 can attenuate IL-6 induced STAT3 activation and increases expression of pro-apoptotic genes (Dimberg *et al.*, 2012). These STAT proteins also have contrasting effects on cancer immunity and inflammation. STAT1 is mainly activated through interferon (α, β, and γ) signalling and results in the expression of T_H1 immunostimulatory and pro-apoptotic genes (Yu *et al.*, 2009). Interleukins-6, 10, 11, 21, and 23,

stimulate STAT3 and increases expression of T_H17 type genes, which are involved in the inflammatory response (Yu *et al.*, 2009).

Changes in *STAT1* gene expression in cell lines treated with ruxolitinib was observed only in those carrying a *JAK2*^{V617F} mutation (Figure 66) suggesting that the reduction in total STAT1 may occur at the transcriptional level, rather than post translational modifications such as increased proteolytic processing.

SOCS proteins are important negative regulators of JAK/STAT signalling and are upregulated in response to cytokine binding in a classical negative feedback loop (Krebs and Hilton, 2001). The activity of SOCS1 is mostly targeted at modulating IFN-γ signalling, whereas IL-6 induction of JAK/STAT is primarily regulated by SOCS3. Both of these inhibitors have different mechanisms in how they negatively regulate JAK/STAT. SOCS1 binds directly to activated JAK2 at the autophosphorylation site (1001-1013) and targets it for ubiquitin-dependent proteolytic degradation (Ungureanu *et al.*, 2002, Waiboci *et al.*, 2007). SOCS3 mechanism is slightly different, it can bind simultaneously to JAK2 as well as to the membrane bound receptor, catalysing the ubiquitination of both (Babon *et al.*, 2012). SOCS3 can directly inhibit JAK proteins in the absence of receptor binding, although this is with much poorer affinity than SOCS1 (Babon *et al.*, 2012). When SET2 cells were exposed to ruxolitinib, contrasting results were observed between expression levels of *SOCS1* and *SOCS3* (Figure 73). Despite increased levels of transcription for genes encoding the pro-inflammatory and STAT3 stimulating IL-6 and IL-1b (Samavati *et al.*, 2009), there was a twofold decrease in expression of *SOCS3. SOCS1* did not decrease, with levels increasing 1.5-fold. One potential explanation for these results may lie in the previously described binding mechanisms for SOCS1 and SOCS3, as well as the paradoxical phosphorylation of JAK2 in the presence of ruxolitinib. Both JAK2 and membrane receptor activation in proximity are needed to stimulate SOCS3 (Babon *et al.*, 2012), and this is blocked with ruxolitinib. SOCS1, however, is primarily induced by JAK2 activation alone, which occurs due to autophosphorylation of Tyr1007/1008 in the activation loop (Andraos *et al.*, 2012).

Ruxolitinib was compared to the STAT1 specific inhibitor, fludarabine. Fludarabine reduces STAT1 phosphorylation, mRNA and protein expression, but does not affect the levels of other STAT proteins, including STAT3 and STAT5 (Frank *et al.*, 1999). JAK2 phosphorylation is also unaffected, although total levels of JAK2 decrease 5 days after STAT1 inhibition, indicating that total JAK2 suppression is secondary to anti-STAT1 activity (Torella *et al.*, 2007). SET2 was less sensitive to fludarabine (IC_{50} = 5.5 µM) (Figure 74) compared to ruxolitinib (IC_{50} = 132 nM) (Figure 48) in cell proliferation assays. Ruxolitinib was also a more potent inhibitor of *STAT1* gene expression, treatment with 1 µM RUX resulted in a three-fold reduction of *STAT1* in SET2 cells (Figure 75). Fludarabine dosed at a greater concentration (5.5 µM) only resulted in a downregulation of *STAT1* by 30%. These results suggest that targeting JAK2 inhibition, upstream from STAT1 signalling, has a greater effect on cell proliferation and STAT1 expression than targeting STAT1 directly.

These experiments highlight the crucial role that STAT1 plays in modulating JAK signalling in MPNs. Differential expression of STAT proteins, including STAT1, and responses to JAK inhibitors are important factors in predicting how MPNs may progress and respond to treatment. Interactions with other molecular pathways, including immunity and inflammatory responses (modulated by STAT1) are significant considerations when examining potential treatment side-effects during targeted JAK/STAT inhibition.

CHAPTER 6: Overview

Knowledge and understanding of the molecular mechanisms underpinning the *BCR-ABL* negative myeloproliferative neoplasms has improved greatly in the last 12 years. The discovery of the *JAK2*^{V617F} mutation in MPN patients (Baxter *et al.*, 2005, James *et al.*, 2005, Kralovics *et al.*, 2005, Levine *et al.*, 2005) has been perhaps the most important advance since the classification of these disorders by Dameshek in the 1950s (Dameshek, 1951). More recently, the identification of calreticulin mutations in ET and MF has provided an almost complete picture of the genetic landscape of MPN (Klampfl *et al.*, 2013, Nangalia *et al.*, 2013). Regardless of differences in phenotype or mutation type, dysregulation of the JAK/STAT pathway is a shared feature. Therefore, this project aimed to investigate what impact changes at a nuclear level (independent of JAK2) had in MPNs, as well as investigating whether these changes affected disease pathogenesis. Global proteomic changes and modifications in ruxolitinib treated cells were also examined in order to determine the impact of JAK2 dysregulation as well as identifying potential pathways for treatment.

Changes at the nuclear level, affecting transcription of critical haematopoietic transcription factors, were studied in ET patients. The haematopoietic transcription factor, GATA1, is a key modulator of cell differentiation and lineage commitment (Crispino, 2005). It was found that *GATA1* was significantly upregulated in these patients, which was independent of mutational status. Notably, the correlation with one of the clinicopathological indicators for ET, platelet counts, was negative.

To further investigate the role of *GATA1* expression, changes in MPN cell line models were studied using anagrelide. The rationale for selecting this drug were; 1) its use as a platelet-reducing agent in ET, 2) it was not known to affect JAK/STAT pathway signalling, and 3) previous experiments have shown a potential link between *GATA1* downregulation and the mechanism of this drug. Therefore, changes elicited in cell proliferation, cell cycle stages, and gene expression would be independent from JAK/STAT signalling, but instead result from *GATA1* downregulation. Haematopoietic gene expression, including *GATA1*, was not significantly affected by anagrelide treatment but the *JAK2*^{V617F} cell line, HEL, responded to the drug in cellular proliferation assays. This cell line was derived from a patient with no

known history of MPN, but with the *JAK2*^{V617F} mutation. Whilst proliferation was reduced, and evidence of a block in cell cycle progression from G1 to S phase was shown, this did not impact on the expression of key haematopoietic genes in this cell line. GATA1 has multifunctional roles, often dependent on presence of co-factors expressed at different stages of development (Chang *et al.*, 2002a, Crispino, 2005). In the experiments by Ahluwalia *et al.* (2010), *GATA1* downregulation in response to anagrelide was shown in primary cells during TPO-induced differentiation. Therefore, to simulate the effect of *GATA1* downregulation during megakaryocyte differentiation, the phorbol ester, PMA, was used. Whilst *GATA1* expression was unchanged, transcription of genes involved in megakaryocyte differentiation under GATA1 transcriptional control were affected. It is not known whether these results are specific to this cell line.

Chapters 3 and 4 investigated nuclear changes downstream and independent from JAK/STAT signalling. Chapter 5 examined how the JAK/STAT pathway is directly responsible for global proteomic changes as well as functional protein modifications. Ruxolitinib, the first JAK inhibitor drug to be licensed for use in MPN patients, was used to target JAK/STAT signalling. At concentrations up to 1 μM, this drug was selective (inhibiting cell proliferation) against those cell lines containing the *JAK2*^{V617F} mutation. Despite this apparent specificity, there is no complete reduction in the clonal burden in patients treated with ruxolitinib (Verstovsek *et al.*, 2014). In clinical trials, it has been shown to be equally effective against both *JAK2*-mutated and non-*JAK2*-mutated patients, indicating that all mutations found in MPN result in dysregulated signalling via JAK2/STAT pathways (Verstovsek *et al.*, 2014). Targeted drugs against JAK2 should therefore, in theory, be effective against all MPNs. However in the *CALR*-mutated MARIMO, a cell line derived from an AML patient with previous history of ET, ruxolitinib does not have any effect on proliferation (Kollmann *et al.*, 2015). This group have also shown that the cell line does not depend on JAK2/STAT5 signalling and *CALR* expression is 10-fold higher than seen in *JAK2*^{V617F} cell lines. The results in this study (Chapter 3) indicate that downregulation of *CALR* is common to all MPNs, although proteomics in Chapter 5 did not show any significant reduction in calreticulin protein levels.

Phosphorylated levels of all the STAT proteins (1/3/5) were decreased with ruxolitinib treatment in *JAK2*^{V617F} cell lines. Interestingly total STAT1 levels also decreased, a finding confirmed with global proteomics studies. Gene expression experiments suggest that downregulation of *STAT1* induced by ruxolitinib may be responsible, but decreased protein synthesis or increased proteolytic degradation may also play a role.

The enhanced effectiveness of ruxolitinib in *STAT1* inhibition compared to fludarabine in the SET2 cell line was demonstrated. As fludarabine is associated with significant immunosuppressive activity, these results should highlight the importance of monitoring patients undergoing ruxolitinib therapy. Indeed, there have already been several case reports where ruxolitinib treatment has been associated with opportunistic viral and intracellular infections (Wysham *et al.*, 2013, von Hofsten *et al.*, 2016, Heine *et al.*, 2013). It is not yet known whether other JAK inhibitors undergoing clinical trials will inhibit STAT1 in a similar manner as ruxolitinib. As more tyrosine kinase inhibitors are developed which seek to target JAK/STAT signalling in MPN, there will be a greater need to understand differential STAT1 responses to these drugs.

CHAPTER 7: Conclusions & future work

7.1: Conclusions:

This project has demonstrated that *GATA1* may have potential as a biomarker in ET, independent from mutational or treatment status. This was shown in PBMCs isolated from MPN donor samples. Experiments utilising model JAK2 cell lines then showed that pathways directly linked to GATA1 could regulate gene expression involved in terminal megakaryopoiesis, which has implications for the disease and the mechanism of anagrelide. Direct JAK2 inhibition, in both cell line models and patient samples, highlighted the importance of STAT1, in MPN disease pathogenesis as well as potential implications in immune responses arising from the use of JAK/STAT inhibitors.

7.2: Future Work:

Monitoring of patient platelet levels and disease progression over time along with *GATA1* expression may determine whether *GATA1* has potential as a surrogate diagnostic and/or prognostic biomarker for the disorder. The recently discovered mechanism by which mutant CALR interacts with the TPO receptor (MPL) should also be studied to determine whether there is any link between this and the decreased CALR expression observed in my studies. Although decreased *CALR* gene expression was observed across all ET disease mutations, it is not known whether this is reflected in the CALR protein levels and whether there are changes in the proportion of mutant calreticulin located in the ER. As with *GATA1*, it may be worth examining how *CALR* expression levels vary during the progression of these disorders and whether this has any prognostic or diagnostic significance.

Primary cell lines should also be used to confirm whether changes in *GATA1* expression levels lead to changes downstream of genes involved in regulating megakaryopoiesis, as observed in experiments with cultured cell lines. Further experiments using primary cell lines undergoing megakaryocytic differentiation in the presence of anagrelide should be carried out to confirm these findings. Gene expression experiments using other PDE III inhibitors during induced megakaryopoiesis may also demonstrate whether this particular mechanism is responsible for the reduction of *PSTPIP2* and *PF4.*

Proteomic studies should be carried out, using cell models where JAK2 and STAT1 are induced to overexpress, to complement the studies here where JAK2 has been negatively regulated by ruxolitinib. More patient samples are required to confirm the differential STAT1 protein expression patterns observed between ET and PV patients. Further work examining patient to patient variations in STAT1 expression, and any changes during the course of the disease would also be valuable. These studies may help to predict the likely response to ruxolitinib therapy, side effects, and whether patient is at greater risk of disease transformation.

CHAPTER 8: Appendices

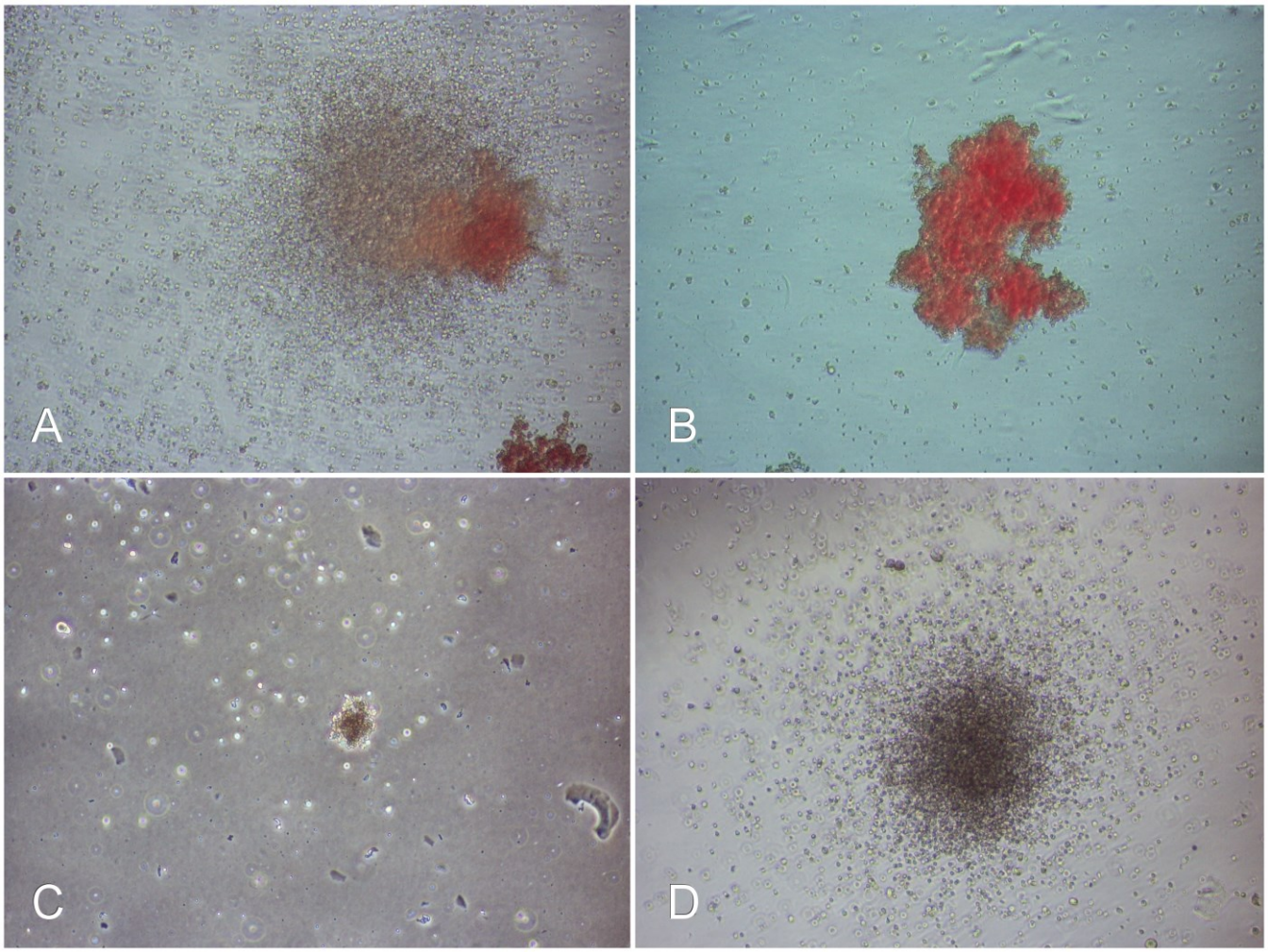


Figure 76: Representative images of A) CFU-GEMM, B) BFU-E, C) CFU-E and D) CFU-GM from colony forming unit assays. Images were taken after PBMCs were cultured for 14 days in methylcelluose media

Table 13: Differential protein expression (< 0.8) measured by iTRAQ in SET2 cells treated with 1 µM ruxolitinib.

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9P1F3	ABRACL	ABRA C-terminal like(ABRACL)	2.58	0.0105	0.0172	0.0540	0.1320	0.0105	0.0172	**0.0105**	**0.0172**
E9PNC7	DRAP1	DR1 associated protein 1(DRAP1)	2.01	0.0106	0.0186	0.0773	0.1502	0.0106	0.0186	**0.0106**	**0.0186**
Q8WTS1	ABHD5	abhydrolase domain containing 5(ABHD5)	4	0.0111	0.0232	1.0280	0.9462	0.0111	0.0232	**0.0111**	**0.0232**
Q9BR61	ACBD6	acyl-CoA binding domain containing 6(ACBD6)	2.7	0.0111	0.0207	0.3981	0.2553	0.0111	0.0207	**0.0111**	**0.0207**
O43747	AP1G1	adaptor related protein complex 1 gamma 1 subunit(AP1G1)	2	0.0111	0.0228	0.0111	0.0228	0.0111	0.0228	**0.0111**	**0.0228**
O95782	AP2A1	adaptor related protein complex 2 alpha 1 subunit(AP2A1)	6.2	0.0111	0.0229	0.4406	0.2861	0.0111	0.0229	**0.0111**	**0.0229**
P02649	APOE	apolipoprotein E(APOE)	2.03	0.4529	0.2945	0.0111	0.0212	0.0111	0.0212	**0.0111**	**0.0212**
Q15392	DHCR24	24-dehydrocholesterol reductase(DHCR24)	2.14	0.0111	0.0211	0.0111	0.0211	0.2884	0.1961	**0.0111**	**0.0211**
Q9H6R0	DHX33	DEAH-box helicase 33(DHX33)	4.28	0.0111	0.0231	0.2148	0.1599	0.0111	0.0231	**0.0111**	**0.0231**
H0YK61	EMC4	ER membrane protein complex subunit 4(EMC4)	4	0.0111	0.0226	0.4246	0.2762	0.0111	0.0226	**0.0111**	**0.0226**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
H3BS72	HACD3	3-hydroxyacyl-CoA dehydratase 3(HACD3)	1.35	0.0111	0.0206	0.4966	0.3392	0.0111	0.0206	**0.0111**	**0.0206**
P53701	HCCS	holocytochrome c synthase(HCCS)	2.11	0.0111	0.0207	0.8166	0.7359	0.0111	0.0207	**0.0111**	**0.0207**
P31937	HIBADH	3-hydroxyisobutyrate dehydrogenase(HIBADH)	3.86	0.0111	0.0231	1.1169	0.8158	0.0111	0.0231	**0.0111**	**0.0231**
Q53FT3	HIKESHI	Hikeshi, heat shock protein nuclear import factor(HIKESHI)	4	0.0111	0.0219	0.0111	0.0219	0.2377	0.1700	**0.0111**	**0.0219**
Q14145	KEAP1	kelch like ECH associated protein 1(KEAP1)	2.37	0.0111	0.0229	0.0111	0.0229	0.0111	0.0229	**0.0111**	**0.0229**
O14733	MAP2K7	mitogen-activated protein kinase kinase 7(MAP2K7)	4.38	0.0111	0.0233	1.5136	0.4744	0.0111	0.0233	**0.0111**	**0.0233**
Q9Y316	MEMO1	mediator of cell motility 1(MEMO1)	4	0.0111	0.0210	0.0111	0.0210	0.9204	0.8784	**0.0111**	**0.0210**
Q9Y483	MTF2	metal response element binding transcription factor 2(MTF2)	2.01	0.0111	0.0233	1.3183	0.5976	0.0111	0.0233	**0.0111**	**0.0233**
Q8IXK0	PHC2	polyhomeotic homolog 2(PHC2)	4.01	1.0471	0.9108	0.0111	0.0223	0.0111	0.0223	**0.0111**	**0.0223**
Q9HCU5	PREB	prolactin regulatory element binding(PREB)	2.02	0.0111	0.0227	0.5970	0.4167	0.0111	0.0227	**0.0111**	**0.0227**
Q9UNN8	PROCR	protein C receptor(PROCR)	3.04	0.0111	0.0206	0.0809	0.2434	0.0111	0.0206	**0.0111**	**0.0206**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
H0Y8D1	PRSS1	protease, serine 1(PRSS1)	2.78	0.0111	0.0184	0.0111	0.0184	0.0111	0.0184	**0.0111**	**0.0184**
Q5T8U5	SURF4	surfeit 4(SURF4)	2	0.0111	0.0195	0.0560	0.1540	0.0111	0.0195	**0.0111**	**0.0195**
J3QQW9	SUZ12	SUZ12 polycomb repressive complex 2 subunit(SUZ12)	4.44	0.0111	0.0222	0.9638	0.9619	0.0111	0.0222	**0.0111**	**0.0222**
P49754	VPS41	VPS41, HOPS complex subunit(VPS41)	2.11	0.0111	0.0206	0.0111	0.0206	0.0111	0.0206	**0.0111**	**0.0206**
Q9UIV1	CNOT7	CCR4-NOT transcription complex subunit 7(CNOT7)	1.77	9.0365	0.1202	0.0127	0.0226	0.0127	0.0226	**0.0127**	**0.0226**
O00623	PEX12	peroxisomal biogenesis factor 12(PEX12)	2.03	11.8032	0.0999	0.0129	0.0255	0.0129	0.0255	**0.0129**	**0.0255**
P69891	HBG1	hemoglobin subunit gamma 1(HBG1)	2	0.0138	0.0171	0.0402	0.1348	0.0138	0.0171	**0.0138**	**0.0171**
Q9UMX0	UBQLN1	ubiquilin 1(UBQLN1)	10	0.0194	0.0350	0.0863	0.1615	0.0203	0.0351	**0.0203**	**0.0351**
O75122	CLASP2	cytoplasmic linker associated protein 2(CLASP2)	8.13	0.0111	0.0230	0.0223	0.0343	0.4130	0.0532	**0.0223**	**0.0343**
P18085	ARF4	ADP ribosylation factor 4(ARF4)	3.91	0.1047	0.0354	0.0254	0.0066	0.0180	0.0054	**0.0254**	**0.0066**
Q9BWJ5	SF3B5	splicing factor 3b subunit 5(SF3B5)	2.86	0.0275	0.0351	0.0294	0.0358	0.0305	0.0361	**0.0294**	**0.0358**
Q8TDB8	SLC2A14	solute carrier family 2 member 14(SLC2A14)	4.29	0.0316	0.0383	0.0334	0.0389	0.0281	0.0370	**0.0316**	**0.0383**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9Y5M8	SRPRB	SRP receptor beta subunit(SRPRB)	5.1	0.0231	0.0136	0.0316	0.0325	0.0540	0.0237	**0.0316**	**0.0237**
G3V5Q1	APEX1	apurinic/apyrimidinic endodeoxyribonuclease 1(APEX1)	4.68	0.0337	0.0398	0.1343	0.2702	0.0316	0.0395	**0.0337**	**0.0398**
P63244	RACK1	receptor for activated C kinase 1(RACK1)	16.8	0.0377	0.0108	0.0560	0.0250	0.0360	0.0079	**0.0377**	**0.0108**
Q9BPX3	NCAPG	non-SMC condensin I complex subunit G(NCAPG)	8.04	0.0384	0.0012	0.0832	0.0038	0.0363	0.0012	**0.0384**	**0.0012**
Q15269	PWP2	periodic tryptophan protein homolog (yeast)(PWP2)	5.54	0.0402	0.0397	0.0863	0.1500	0.0136	0.0330	**0.0402**	**0.0397**
C9JVN9	L2HGDH	L-2-hydroxyglutarate dehydrogenase(L2HGDH)	6	0.0425	0.0393	0.5495	0.3982	0.0406	0.0388	**0.0425**	**0.0393**
P00352	ALDH1A1	aldehyde dehydrogenase 1 family member A1(ALDH1A1)	5.19	0.0466	0.0179	0.1722	0.0493	0.0394	0.0199	**0.0466**	**0.0199**
O95714	HERC2	HECT and RLD domain containing E3 ubiquitin protein ligase 2(HERC2)	8.42	0.0474	0.0431	0.1406	0.2410	0.0398	0.0412	**0.0474**	**0.0431**
Q9NUQ2	AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5(AGPAT5)	2.14	0.0483	0.0348	0.0384	0.0362	0.3981	0.3551	**0.0483**	**0.0362**
Q15637	SF1	splicing factor 1(SF1)	11.52	0.0492	0.1844	0.7656	0.0485	0.0238	0.0356	**0.0492**	**0.0485**
Q13616	CUL1	cullin 1(CUL1)	4.19	0.2938	0.0054	0.0535	0.0207	0.0107	0.0006	**0.0535**	**0.0054**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9BYD3	MRPL4	mitochondrial ribosomal protein L4(MRPL4)	4	0.0182	0.0220	0.2992	0.3884	0.0570	0.0392	**0.0570**	**0.0392**
Q9Y512	SAMM50	SAMM50 sorting and assembly machinery component(SAMM50)	3.23	0.0570	0.0398	0.1923	0.1639	0.0363	0.0377	**0.0570**	**0.0398**
O00764	PDXK	pyridoxal (pyridoxine, vitamin B6) kinase(PDXK)	5.05	0.0586	0.0429	0.4742	0.3344	0.0560	0.0424	**0.0586**	**0.0429**
P62891	RPL39	ribosomal protein L39(RPL39)	4.11	0.0637	0.0037	1.1376	0.1743	0.0483	0.0032	**0.0637**	**0.0037**
Q9Y277	VDAC3	voltage dependent anion channel 3(VDAC3)	9.46	0.0377	0.0059	0.0718	0.2028	0.0649	0.0007	**0.0649**	**0.0059**
Q969X6	UTP4	UTP4, small subunit processome component(UTP4)	1.77	0.1854	0.0413	0.0340	0.0228	0.0661	0.0228	**0.0661**	**0.0228**
P05387	RPLP2	ribosomal protein lateral stalk subunit P2(RPLP2)	13.41	0.0530	0.0242	0.1820	0.0005	0.0685	0.0305	**0.0685**	**0.0242**
J3KSZ5	DCXR	dicarbonyl and L-xylulose reductase(DCXR)	6.02	0.2249	0.0061	0.0109	0.0007	0.0724	0.0032	**0.0724**	**0.0032**
Q8NE86	MCU	mitochondrial calcium uniporter(MCU)	2	0.0780	0.0217	0.0780	0.0217	0.8091	0.6450	**0.0780**	**0.0217**
Q86UX7	FERMT3	fermitin family member 3(FERMT3)	34.81	0.0817	0.0026	0.1271	0.0072	0.0437	0.0015	**0.0817**	**0.0026**
P31946	YWHAB	tyrosine 3-monooxygenase/tryptophan 5-	4.94	0.0912	0.0347	0.1644	0.0896	0.0938	0.0327	**0.0938**	**0.0347**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
		monooxygenase activation protein beta(YWHAB)									
Q14376	GALE	UDP-galactose-4-epimerase(GALE)	4.85	0.2148	0.1697	0.0912	0.0399	0.0991	0.0409	**0.0991**	**0.0409**
Q9Y6K5	OAS3	2'-5'-oligoadenylate synthetase 3(OAS3)	2.03	0.1009	0.0458	0.0766	0.0433	0.1076	0.0463	**0.1009**	**0.0458**
Q9BQ52	ELAC2	elaC ribonuclease Z 2(ELAC2)	8.92	0.0111	0.0236	0.1019	0.0440	0.1107	0.0447	**0.1019**	**0.0440**
Q05086	UBE3A	ubiquitin protein ligase E3A(UBE3A)	4.18	0.8630	0.7841	0.1019	0.0448	0.1009	0.0447	**0.1019**	**0.0448**
Q15102	PAFAH1B3	platelet activating factor acetylhydrolase 1b catalytic subunit 3(PAFAH1B3)	6.16	0.0310	0.0019	0.3733	0.0674	0.1028	0.0120	**0.1028**	**0.0120**
O75964	ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex subunit G(ATP5L)	6.2	0.0331	0.0176	0.1038	0.0365	0.1854	0.0651	**0.1038**	**0.0365**
B0I1T2	MYO1G	myosin IG(MYO1G)	9.2	0.1038	0.0423	0.0817	0.0440	0.1047	0.0423	**0.1038**	**0.0423**
P61086	UBE2K	ubiquitin conjugating enzyme E2 K(UBE2K)	6.41	0.1096	0.0197	0.3373	0.0884	0.0780	0.0266	**0.1096**	**0.0266**
P00491	PNP	purine nucleoside phosphorylase(PNP)	14.2	0.1127	0.0368	0.1076	0.0327	0.1803	0.0712	**0.1127**	**0.0368**
I3L0U5	CCDC137	coiled-coil domain containing 137(CCDC137)	4	0.0982	0.0458	0.1236	0.0453	0.1159	0.0447	**0.1159**	**0.0453**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9H6D7	HAUS4	HAUS augmin like complex subunit 4(HAUS4)	1.49	0.2249	0.9671	0.0370	0.0327	0.1236	0.0435	**0.1236**	**0.0435**
Q08211	DHX9	DExH-box helicase 9(DHX9)	37.96	0.1294	0.0094	0.2489	0.0088	0.1294	0.0128	**0.1294**	**0.0094**
P82930	MRPS34	mitochondrial ribosomal protein S34(MRPS34)	3.26	0.0817	0.0387	0.1306	0.0448	0.1318	0.0455	**0.1306**	**0.0448**
O15144	ARPC2	actin related protein 2/3 complex subunit 2(ARPC2)	9.55	0.1047	0.0419	0.3802	0.1331	0.1380	0.0042	**0.1380**	**0.0419**
Q9H7M9	VSIR	V-set immunoregulatory receptor(VSIR)	2.05	0.0363	0.0386	0.3467	0.2495	0.1393	0.0427	**0.1393**	**0.0427**
Q9Y3T9	NOC2L	NOC2 like nucleolar associated transcriptional repressor(NOC2L)	6.03	0.1419	0.0448	0.2312	0.0518	0.1047	0.0461	**0.1419**	**0.0461**
P51149	RAB7A	RAB7A, member RAS oncogene family(RAB7A)	15.52	0.1330	0.0191	0.1419	0.0109	0.2208	0.0479	**0.1419**	**0.0191**
I3L1P8	SLC25A11	solute carrier family 25 member 11(SLC25A11)	3.08	0.0839	0.0435	0.4742	0.3309	0.1472	0.0480	**0.1472**	**0.0480**
P55884	EIF3B	eukaryotic translation initiation factor 3 subunit B(EIF3B)	24.19	0.1500	0.0153	0.2421	0.0727	0.1247	0.0093	**0.1500**	**0.0153**
A6NJA2	USP14	ubiquitin specific peptidase 14(USP14)	8.52	0.1528	0.0373	0.0213	0.0203	0.3281	0.1835	**0.1528**	**0.0373**
C9JFR7	CYCS	cytochrome c, somatic(CYCS)	4.36	0.0863	0.0219	0.4093	0.2869	0.1542	0.0341	**0.1542**	**0.0341**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
O95563	MPC2	mitochondrial pyruvate carrier 2(MPC2)	1.55	0.1600	0.0405	0.2168	0.1754	0.1472	0.0401	**0.1600**	**0.0405**
Q9Y6C9	MTCH2	mitochondrial carrier 2(MTCH2)	8.01	0.2466	0.0689	0.1127	0.0410	0.1629	0.0361	**0.1629**	**0.0410**
P41252	IARS	isoleucyl-tRNA synthetase(IARS)	26.08	0.2489	0.0041	0.1644	0.0003	0.0904	0.0171	**0.1644**	**0.0041**
P46977	STT3A	STT3A, catalytic subunit of the oligosaccharyltransferase complex(STT3A)	5.22	0.1556	0.0477	0.2489	0.0651	0.1644	0.0439	**0.1644**	**0.0477**
Q86VP6	CAND1	cullin associated and neddylation dissociated 1(CAND1)	14.33	0.1854	0.0003	0.1690	0.0047	0.1294	0.0213	**0.1690**	**0.0047**
O43264	ZW10	zw10 kinetochore protein(ZW10)	4.9	0.1820	0.0043	0.1722	0.0904	0.1009	0.0391	**0.1722**	**0.0391**
Q9NVI7	ATAD3A	ATPase family, AAA domain containing 3A(ATAD3A)	25	0.1820	0.0002	0.2606	0.0002	0.1419	0.0455	**0.1820**	**0.0002**
P57740	NUP107	nucleoporin 107(NUP107)	8.68	0.1854	0.0051	0.4365	0.2990	0.1393	0.0061	**0.1854**	**0.0061**
A6NFX8	NUDT5	nudix hydrolase 5(NUDT5)	7.67	0.1977	0.1476	0.2884	0.0358	0.1977	0.0109	**0.1977**	**0.0358**
Q9NVI1	FANCI	Fanconi anemia complementation group I(FANCI)	3.55	0.6427	0.2721	0.1803	0.0048	0.2014	0.0056	**0.2014**	**0.0056**
Q15393	SF3B3	splicing factor 3b subunit 3(SF3B3)	21.92	0.2188	0.0869	0.2014	0.0086	0.1225	0.0162	**0.2014**	**0.0162**
P47897	QARS	glutaminyl-tRNA synthetase(QARS)	33.72	0.1786	0.0080	0.4093	0.0081	0.2089	0.0079	**0.2089**	**0.0080**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q5SZR4	TDRKH	tudor and KH domain containing(TDRKH)	2.03	0.4742	0.1446	0.2148	0.0226	0.2070	0.0226	**0.2148**	**0.0226**
Q13200	PSMD2	proteasome 26S subunit, non-ATPase 2(PSMD2)	17.78	0.4285	0.0251	0.2168	0.0530	0.1459	0.0167	**0.2168**	**0.0251**
Q15020	SART3	squamous cell carcinoma antigen recognized by T-cells 3(SART3)	14.66	0.3698	0.0193	0.2228	0.0166	0.0832	0.0872	**0.2228**	**0.0193**
Q9NZ45	CISD1	CDGSH iron sulfur domain 1(CISD1)	4.15	0.1472	0.0108	0.2512	0.0250	0.2249	0.0254	**0.2249**	**0.0250**
P00338	LDHA	lactate dehydrogenase A(LDHA)	19.36	0.2249	0.0531	0.2399	0.0137	0.0912	0.0131	**0.2249**	**0.0137**
P55060	CSE1L	chromosome segregation 1 like(CSE1L)	24.03	0.2070	0.0367	0.3873	0.0003	0.2270	0.0242	**0.2270**	**0.0242**
Q92769	HDAC2	histone deacetylase 2(HDAC2)	13.68	0.2188	0.0055	0.5916	0.2198	0.2291	0.0056	**0.2291**	**0.0056**
P14868	DARS	aspartyl-tRNA synthetase(DARS)	25.02	0.2312	0.0112	0.3597	0.0273	0.1472	0.0962	**0.2312**	**0.0273**
O95870	ABHD16A	abhydrolase domain containing 16A(ABHD16A)	6.12	0.7311	0.1746	0.2333	0.0062	0.2249	0.0064	**0.2333**	**0.0064**
Q9NQ55	PPAN	peter pan homolog (Drosophila)(PPAN)	2.06	0.1169	0.0223	0.3733	0.0868	0.2333	0.0402	**0.2333**	**0.0402**
P08240	SRPRA	SRP receptor alpha subunit(SRPRA)	19.52	0.1941	0.0129	0.3597	0.0432	0.2333	0.0713	**0.2333**	**0.0432**
P04818	TYMS	thymidylate synthetase(TYMS)	8	0.2355	0.0249	0.5248	0.1973	0.0661	0.0126	**0.2355**	**0.0249**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9BQG0	MYBBP1A	MYB binding protein 1a(MYBBP1A)	15.81	0.2421	0.0187	0.3499	0.0833	0.2355	0.0212	**0.2421**	**0.0212**
P41091	EIF2S3	eukaryotic translation initiation factor 2 subunit gamma(EIF2S3)	14.19	0.3105	0.0025	0.2443	0.0022	0.2051	0.0029	**0.2443**	**0.0025**
P01023	A2M	alpha-2-macroglobulin(A2M)	8.27	0.4365	0.0049	0.2489	0.0005	0.0817	0.0013	**0.2489**	**0.0013**
P62266	RPS23	ribosomal protein S23(RPS23)	6.24	0.2535	0.0554	0.3048	0.0037	0.2377	0.0048	**0.2535**	**0.0048**
P40429	RPL13A	ribosomal protein L13a(RPL13A)	15.21	0.2051	0.1156	0.3873	0.0062	0.2630	0.0058	**0.2630**	**0.0062**
P06744	GPI	glucose-6-phosphate isomerase(GPI)	41.16	0.2655	0.0136	0.3076	0.0470	0.2208	0.0580	**0.2655**	**0.0470**
J9JIE6	TMCO1	transmembrane and coiled-coil domains 1(TMCO1)	4	0.2655	0.0407	0.2466	0.0397	0.3467	0.0414	**0.2655**	**0.0407**
Q6P2Q9	PRPF8	pre-mRNA processing factor 8(PRPF8)	31.89	0.2679	0.0046	0.5012	0.0004	0.2399	0.0632	**0.2679**	**0.0046**
P02774	GC	GC, vitamin D binding protein(GC)	7.5	0.6427	0.0971	0.2858	0.0361	0.2355	0.0026	**0.2858**	**0.0361**
P16435	POR	cytochrome p450 oxidoreductase(POR)	5.83	0.2992	0.0451	1.5417	0.7547	0.3020	0.0451	**0.3020**	**0.0451**
P21796	VDAC1	voltage dependent anion channel 1(VDAC1)	23.01	0.2089	0.0045	0.3373	0.0138	0.3020	0.0143	**0.3020**	**0.0138**
Q10567	AP1B1	adaptor related protein complex 1 beta 1 subunit(AP1B1)	14.59	0.4966	0.0011	0.3020	0.0217	0.3076	0.0017	**0.3076**	**0.0017**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9NYB0	TERF2IP	TERF2 interacting protein(TERF2IP)	3.93	0.3105	0.0490	2.8576	0.3646	0.2704	0.0477	**0.3105**	**0.0490**
P20645	M6PR	mannose-6-phosphate receptor, cation dependent(M6PR)	5.69	0.3192	0.0088	0.3597	0.0115	0.1445	0.0098	**0.3192**	**0.0098**
P45880	VDAC2	voltage dependent anion channel 2(VDAC2)	5.03	0.2754	0.0154	0.3192	0.0161	0.3597	0.0191	**0.3192**	**0.0161**
P13051	UNG	uracil DNA glycosylase(UNG)	6.81	0.3342	0.0482	2.4434	0.9188	0.3404	0.0484	**0.3404**	**0.0484**
H7C2I1	PRMT1	protein arginine methyltransferase 1(PRMT1)	3.84	0.3698	0.0112	0.3565	0.0118	0.1570	0.2385	**0.3565**	**0.0118**
O75165	DNAJC13	DnaJ heat shock protein family (Hsp40) member C13(DNAJC13)	3.93	0.3698	0.0038	0.3837	0.0049	0.8872	0.9262	**0.3837**	**0.0049**
P42566	EPS15	epidermal growth factor receptor pathway substrate 15(EPS15)	7.78	0.4613	0.0534	0.4130	0.0237	0.2911	0.0111	**0.4130**	**0.0237**
B4DWR3	VBP1	VHL binding protein 1(VBP1)	2.15	0.6730	0.0728	0.3499	0.0299	0.4169	0.0406	**0.4169**	**0.0406**
F8VYN9	ARL1	ADP ribosylation factor like GTPase 1(ARL1)	2	0.4487	0.1530	0.4285	0.0429	0.3945	0.0419	**0.4285**	**0.0429**
P49257	LMAN1	lectin, mannose binding 1(LMAN1)	5.71	0.4325	0.0194	0.6668	0.1564	0.3631	0.0133	**0.4325**	**0.0194**
O75643	SNRNP200	small nuclear ribonucleoprotein U5 subunit 200(SNRNP200)	43.52	0.4365	0.0084	0.4207	0.0131	0.4656	0.0058	**0.4365**	**0.0084**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9Y678	COPG1	coatomer protein complex subunit gamma 1(COPG1)	10	0.4487	0.0068	0.4406	0.0069	0.3767	0.0052	**0.4406**	**0.0068**
O43143	DHX15	DEAH-box helicase 15(DHX15)	19.03	0.4406	0.0030	0.4831	0.0162	0.4169	0.0017	**0.4406**	**0.0030**
Q9UL25	RAB21	RAB21, member RAS oncogene family(RAB21)	3.27	0.4406	0.0205	0.4365	0.0205	0.5702	0.0408	**0.4406**	**0.0205**
F8W727	RPL32	ribosomal protein L32(RPL32)	6.19	0.4406	0.0206	0.3436	0.0168	0.4831	0.0989	**0.4406**	**0.0206**
Q9NPA8	ENY2	ENY2, transcription and export complex 2 subunit(ENY2)	4.45	0.4487	0.0414	0.4285	0.0030	0.9204	0.0790	**0.4487**	**0.0414**
Q9UNM6	PSMD13	proteasome 26S subunit, non-ATPase 13(PSMD13)	11.2	0.4786	0.0040	0.6081	0.0063	0.2992	0.1025	**0.4786**	**0.0063**
P51398	DAP3	death associated protein 3(DAP3)	7.68	0.5200	0.0440	0.4875	0.0561	0.3532	0.0287	**0.4875**	**0.0440**
P39656	DDOST	dolichyl-diphosphooligosaccharide--protein glycosyltransferase non-catalytic subunit(DDOST)	12.57	0.4571	0.0125	0.6918	0.0720	0.4875	0.0484	**0.4875**	**0.0484**
P23396	RPS3	ribosomal protein S3(RPS3)	19.62	0.4875	0.0040	0.5105	0.0091	0.4571	0.0050	**0.4875**	**0.0050**
B1ANR0	PABPC4	poly(A) binding protein cytoplasmic 4(PABPC4)	18.35	0.5105	0.0281	0.4966	0.2103	0.3467	0.0303	**0.4966**	**0.0303**
Q01970	PLCB3	phospholipase C beta 3(PLCB3)	9.83	0.5297	0.0468	0.4966	0.0461	0.4966	0.0461	**0.4966**	**0.0461**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q01518	CAP1	adenylate cyclase associated protein 1(CAP1)	16.53	0.5445	0.0125	0.5012	0.0122	0.3837	0.0249	**0.5012**	**0.0125**
Q01813	PFKP	phosphofructokinase, platelet(PFKP)	24.3	0.5248	0.0231	0.5012	0.0068	0.2911	0.0276	**0.5012**	**0.0231**
Q9HB71	CACYBP	calcyclin binding protein(CACYBP)	8.18	0.5495	0.0181	0.5495	0.0238	0.2399	0.1684	**0.5495**	**0.0238**
P42224	STAT1	signal transducer and activator of transcription 1(STAT1)	22.01	0.6792	0.0294	0.5546	0.1963	0.2780	0.0019	**0.5546**	**0.0294**
O00221	NFKBIE	NFKB inhibitor epsilon(NFKBIE)	2.06	0.5649	0.0417	0.5702	0.0419	0.5152	0.0406	**0.5649**	**0.0417**
P11388	TOP2A	topoisomerase (DNA) II alpha(TOP2A)	23.87	0.4487	0.0037	0.6368	0.0057	0.5754	0.0256	**0.5754**	**0.0057**
Q9C0C9	UBE2O	ubiquitin conjugating enzyme E2 O(UBE2O)	10.03	0.5754	0.0051	0.5346	0.0040	0.7047	0.1137	**0.5754**	**0.0051**
E7EUC7	UGP2	UDP-glucose pyrophosphorylase 2(UGP2)	10.12	0.6138	0.0162	0.5754	0.0253	0.5152	0.0119	**0.5754**	**0.0162**
Q99832	CCT7	chaperonin containing TCP1 subunit 7(CCT7)	32.84	0.5808	0.0030	0.5970	0.0230	0.4920	0.0201	**0.5808**	**0.0201**
F1T0B3	DDX1	DEAD-box helicase 1(DDX1)	14.33	0.5808	0.0190	0.5346	0.0340	0.6081	0.0524	**0.5808**	**0.0340**
P42704	LRPPRC	leucine rich pentatricopeptide repeat containing(LRPPRC)	40.71	0.5808	0.0396	0.6138	0.0408	0.4406	0.0179	**0.5808**	**0.0396**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q14149	MORC3	MORC family CW-type zinc finger 3(MORC3)	4.09	0.5395	0.0054	0.6982	0.0317	0.6026	0.0147	**0.6026**	**0.0147**
Q8N2G8	GHDC	GH3 domain containing(GHDC)	2.07	0.6138	0.0435	0.6982	0.3476	0.5754	0.0426	**0.6138**	**0.0435**
P46940	IQGAP1	IQ motif containing GTPase activating protein 1(IQGAP1)	22.02	0.6138	0.0480	0.6730	0.0506	0.4529	0.0333	**0.6138**	**0.0480**
P78527	PRKDC	protein kinase, DNA-activated, catalytic polypeptide(PRKDC)	89.73	0.6138	0.0000	0.6194	0.0000	0.5808	0.0000	**0.6138**	**0.0000**
O00178	GTPBP1	GTP binding protein 1(GTPBP1)	3.88	0.6194	0.0479	0.9727	0.9695	0.5916	0.0473	**0.6194**	**0.0479**
Q71DI3	HIST2H3A	histone cluster 2 H3 family member a(HIST2H3A)	14.79	0.5546	0.0026	0.6486	0.0026	0.6792	0.0028	**0.6486**	**0.0026**
O95864	FADS2	fatty acid desaturase 2(FADS2)	1.9	0.5808	0.0399	0.8241	0.6198	0.6546	0.0422	**0.6546**	**0.0422**
Q92616	GCN1	GCN1, eIF2 alpha kinase activator homolog(GCN1)	59.66	0.7311	0.0275	0.6668	0.0004	0.5152	0.0017	**0.6668**	**0.0017**
P05388	RPLP0	ribosomal protein lateral stalk subunit P0(RPLP0)	24.07	0.7516	0.0032	0.6607	0.0001	0.6668	0.0910	**0.6668**	**0.0032**
O75083	WDR1	WD repeat domain 1(WDR1)	28.74	0.7047	0.0046	0.7311	0.0440	0.6486	0.0147	**0.7047**	**0.0147**
Q9HCS7	XAB2	XPA binding protein 2(XAB2)	3.05	0.5754	0.0029	0.7112	0.0449	0.8395	0.0531	**0.7112**	**0.0449**
P36776	LONP1	lon peptidase 1, mitochondrial(LONP1)	13.47	0.7244	0.0183	0.6918	0.0383	0.8318	0.1162	**0.7244**	**0.0383**

Uniprot code	Gene symbol	Gene name	unused protein score	113 FC	113 p-value	115 FC	115 p-value	117FC	117 p-value	Median fold change (< 0.8)	Median p-value (< 0.05)
Q9UBC3	DNMT3B	DNA methyltransferase 3 beta(DNMT3B)	5.53	0.3698	0.0054	1.2589	0.2856	0.7311	0.0074	**0.7311**	**0.0074**
P06400	RB1	RB transcriptional corepressor 1(RB1)	1.91	1.0000	0.4375	0.7516	0.0330	0.4130	0.0177	**0.7516**	**0.0330**
O95433	AHSA1	activator of Hsp90 ATPase activity 1(AHSA1)	3.43	0.8241	0.0063	0.7656	0.1277	0.6918	0.0079	**0.7656**	**0.0079**
Q00610	CLTC	clathrin heavy chain(CLTC)	59.08	0.7727	0.0021	0.7870	0.0037	0.7727	0.0143	**0.7727**	**0.0037**

Table 14: Gene ontology terms and groupings for downregulated proteins in SET2 cells treated with 1 µM ruxolitinib.

GO: ID	GO: Term	Term PValue Corrected with Bonferroni step down	Group PValue Corrected with Bonferroni step down	GOLevels	GOGroups	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0042987	amyloid precursor protein catabolic process	21.0E-3	2.2E-3	[5, 6]	Group0	13.04	3	[APEX1, APOE, DHCR24]
GO:0018279	protein N-linked glycosylation via asparagine	15.0E-3	2.1E-3	[5, 7, 8, 9]	Group1	8.89	4	[DDOST, LMAN1, PSMD2, STT3A]
GO:0044724	single-organism carbohydrate catabolic process	13.0E-3	2.4E-3	[4, 5]	Group2	4.40	7	[ALDH1A1, DCXR, GALE, GPI, LDHA, NUDT5, PFKP]
GO:0019320	hexose catabolic process	40.0E-3	2.4E-3	[6, 7]	Group2	6.25	4	[ALDH1A1, GALE, GPI, PFKP]
GO:0019080	viral gene expression	2.3E-3	32.0E-6	[5]	Group3	4.48	9	[EIF3B, NUP107, RPL13A, RPL32, RPL39, RPLP0, RPLP2, RPS23, RPS3]

GO: ID	GO: Term	Term PValue Corrected with Bonferroni step down	Group PValue Corrected with Bonferroni step down	GOLevels	GOGroups	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	9.7E-6	32.0E-6	[7, 8, 9, 10, 11, 12]	Group3	8.91	9	[RPL13A, RPL32, RPL39, RPLP0, RPLP2, RPS23, RPS3, SRPRA, SRPRB]
GO:0022618	ribonucleoprotein complex assembly	960.0E-6	220.0E-6	[4, 5, 6]	Group4	4.48	10	[CNOT7, DDX1, EIF3B, LOC102724159, PPAN, PRPF8, RPL13A, SART3, SF1, SNRNP200]
GO:0000245	spliceosomal complex assembly	42.0E-3	220.0E-6	[5, 6, 7, 8, 9, 10, 11, 12]	Group4	5.36	3	[DDX1, SF1, SNRNP200]
GO:0007093	mitotic cell cycle checkpoint	16.0E-3	480.0E-6	[3, 4, 5, 6, 7]	Group5	4.19	7	[CNOT7, FANCI, PRKDC, PRMT1, RB1, TOP2A, ZW10]

GO: ID	GO: Term	Term PValue Corrected with Bonferroni step down	Group PValue Corrected with Bonferroni step down	GOLevels	GOGroups	% Associated Genes	Nr. Genes	Associated Genes Found
GO:1901991	negative regulation of mitotic cell cycle phase transition	4.0E-3	480.0E-6	[5, 6, 7, 8]	Group5	4.13	9	[CNOT7, CUL1, FANCI, PRKDC, PRMT1, PSMD13, PSMD2, RB1, ZW10]
GO:0072431	signal transduction involved in mitotic G1 DNA damage checkpoint	45.0E-3	480.0E-6	[6, 7, 8, 9, 10, 11, 12]	Group5	4.48	3	[CNOT7, PRKDC, PRMT1]
GO:0032770	positive regulation of monooxygenase activity	38.0E-3	2.9E-3	[4, 5, 6]	Group6	10.34	3	[APOE, POR, VDAC2]
GO:0060191	regulation of lipase activity	37.0E-3	2.9E-3	[5]	Group6	4.90	5	[ABHD5, ARF4, ARL1, POR, VDAC2]

GO: ID	GO: Term	Term PValue Corrected with Bonferroni step down	Group PValue Corrected with Bonferroni step down	GOLevels	GOGroups	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0050810	regulation of steroid biosynthetic process	45.0E-3	2.9E-3	[5, 6, 7]	Group6	4.44	4	[APOE, POR, SF1, VDAC2]
GO:0060193	positive regulation of lipase activity	28.0E-3	2.9E-3	[6]	Group6	4.11	3	[ABHD5, ARF4, ARL1]
GO:0016197	endosomal transport	980.0E-6	220.0E-6	[4, 5]	Group7	4.07	11	[AP1G1, AP2A1, ARL1, CLTC, DNAJC13, EPS15, M6PR, RAB21, RAB7A, UBE2O, VPS41]
GO:0016482	cytosolic transport	200.0E-6	220.0E-6	[4, 5]	Group7	6.16	9	[AP1G1, AP2A1, ARL1, CLTC, DNAJC13, EPS15, RAB21, RAB7A, UBE2O]

GO: ID	GO: Term	Term PValue Corrected with Bonferroni step down	Group PValue Corrected with Bonferroni step down	GOLevels	GOGroups	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	34.0E-3	220.0E-6	[5]	Group7	5.15	5	[AP1B1, AP1G1, AP2A1, CLTC, RAB7A]
GO:0032802	low-density lipoprotein particle receptor catabolic process	4.5E-3	220.0E-6	[5, 6, 7]	Group7	23.08	3	[AP2A1, APOE, CLTC]

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21 November 2014

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Dear Dr Graham

Study title:	**Defining and Targeting Myeloproliferative Neoplasms Using Proteomics**
REC reference:	**14/NW/1444**
Protocol number:	**Protocol 1**
IRAS project ID:	**140680**

The Proportionate Review Sub-committee of the NRES Committee North West - Greater Manchester South reviewed the above application on 17 November 2014.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager Nicola Burgess, nrescommittee.northwest-gmsouth@nhs.net.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Summary of discussion at the meeting

Informed consent process and the adequacy and completeness of participant information

The Committee were of the view that the some of the terminology used was slightly complex but did not think it was complex enough to describe as an ethical issue. It was accepted that any terms the participant was not aware of would be explained when required.

Suitability of supporting information

It was noted that the supporting documentation refers too there being 18 participants when there are 24 when the healthy volunteer sample are included. It was agreed that this was not an ethical issue.

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Covering letter on headed paper [Cover Letter]		
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only)		
IRAS Checklist XML [Checklist_07112014]		07 November 2014
Letter from funder		22 May 2014
Other [University of Lincoln Ethical Approval]		
Other [CI CV]		
Other [Health Control PIS]	one	03 September 2014
Other [healthy controls consent form]		
Other [MRC HTA test certificate]		
Other		
Participant consent form	one	17 July 2014
Participant information sheet (PIS)		
REC Application Form [REC_Form_07112014]		07 November 2014
Referee's report or other scientific critique report		
Research protocol or project proposal		
Summary CV for Chief Investigator (CI)		

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

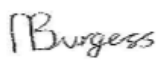
HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at
http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

14/NW/1444	**Please quote this number on all correspondence**

Yours sincerely



PP:
Professor Sobhan Vinjamuri
Chair

Email: nrescommittee.northwest-gmsouth@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

"After ethical review – guidance for researcher

Copy to: *DR ANDREW Stevenson*
Dr Tanweer Ahmed,
UnitedÂ LincolnshireÂ HospitalsÂ NHSÂ Trust,Â LincolnshireÂ ClinicalÂ ResearchÂ Facility

NRES Committee North West - Greater Manchester South

Attendance at PRS Sub-Committee of the REC meeting held in correspondence

Committee Members:

Name	*Profession*	*Present*	*Notes*
Professor Sobhan Vinjamuri (Chair)	Consultant in Nuclear Medicine	Yes	
Dr Anne Armstrong	Consultant Medical Oncologist	Yes	
Mrs Lesley Thornton	Retired Clinical Delivery Director	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Nicola Burgess	CBS Manager / REC Manager

NHS
Health Research Authority

NRES Committee East Midlands - Nottingham 1
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839368
Facsimile: 0115 8839294

16 October 2012

Dr Ciro R Rinaldi
United Lincolnshire Hospital NHS Trust
Haematology Department
Sibsey Road
Boston
UK
PE 21 9QS

Dear Dr Rinaldi

Study title:	**GATA1 Expression levels in peripheral blood of patients with essential thrombocytaemia: implication for pathogenesis and treatment**
IRAS project reference:	**89190**
REC reference:	**12/EM/0350**

The Research Ethics Committee reviewed the above application at the meeting held on 09 October 2012. Thank you for attending to discuss the study.

Ethical opinion

1. The Committee asked you to explain why blood samples are taken before consent has been obtained from the participant. You clarified that the patient will be giving blood routinely, and the research team feel it is convenient for the patient to provide extra blood via the same needle, at the same time. You clarified that the sample will be discarded if the patient does not want to take part in the study.
2. The Committee informed you that it would be preferable to provide the Participant Information Sheet in advance of the blood sample being taken.
3. The Committee informed you that the lay summary provided in the application form is complicated. You gave the Committee an overview of the study.
4. The Committee asked where controls will be recruited from. You informed the Committee it may be the research team or normal blood samples may be used.
5. You clarified participants' GPs will be informed of their participation in the study.
6. The Committee asked if patients will have received treatment for Essential Thrombocytaemia. You explained initial treatment is without intervention, when treatment is started another sample is collected for comparison.
7. You clarified that there are approximately 140 patients in the registry, not all will be taking part in the study but this is the population looked at for this study.
8. The Committee asked if there is any data on Hydroxycarbamide. You informed the Committee that a previous study has been carried out on a small number of samples but more data should be collected for more consistent results.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

1. The Participant Information Sheet should be provided to potential participants with their routine clinic appointment letter.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You must notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Evidence of insurance or indemnity	Policy Numner SZ21693326	
Investigator CV	Dr Ciro Rinaldi	23 August 2012
Other: 24 Hour Patient Confirmation Sheet	1	27 January 2012
Participant Consent Form	1	

Participant Information Sheet	1.0	27 January 2012
Protocol	2	27 January 2012
REC application	89190/357155/1/364	24 August 2012
Referees or other scientific critique report	Dr Prangnell	01 February 2012
Referees or other scientific critique report	N Russell	29 February 2012

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/EM/0350 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp L Gregory

Mr Robert Johnson
Chair

Email: lisa.gregory@nottspct.nhs.uk

Enclosures:	*List of names and professions of members who were present at the meeting and those who submitted written comments* *"After ethical review – guidance for researchers" [SL-AR2]*
Copy to:	*Dr Libby John* *Dr Tanweer Ahmed, United Lincolnshire Hospitals NHS Trust,* *Lincolnshire Clinical Research Facility*

NRES Committee East Midlands - Nottingham 1

Attendance at Committee meeting on 09 October 2012

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Walter Bouman	Consultant Psychiatrist	No	
Dr Glenys Caswell	Research Fellow	Yes	
Professor Cris Constantinescu	Professor of Clinical Neurology	Yes	
Ms Helen Crow	Research Midwife	Yes	
Dr Ursula Holdsworth	Retired Staff Grade Community Paediatrician	Yes	
Mr Robert Johnson (Chair)	Nurse	Yes	
Reverend Keith Lackenby	Lay member	Yes	
Mrs Sarah Lennon	Surgical Registrar GMC registration maintained	Yes	
Mr Jon Merrills	Barrister / Pharmacist	Yes	
Mr Robert Oldroyd	Lay member	Yes	
Dr Noble Philips	General Practitioner	Yes	
Dr Ian Ross	Consultant Physician	Yes	
Mr Ian Thompson	Lay member	Yes	
Mrs Shirley E White	Lay member	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mrs Lisa Gregory	REC Coordinator

CHAPTER 9: References

9.1: Abstracts & Posters

American Society of Mass Spectroscopy (ASMS) 2015

Quantitative proteomic study of the action of ruxolitinib, a potent JAK inhibitor.

Alfonsina D'amato[1]; J.P. Lally[2]; C.R. Rinaldi[2,3]; Robert L. J. Graham[1]; Ciaren Graham[2]

1: University of Manchester, Manchester, UK; 2: School of Life Sciences, University of Lincoln, Lincoln UK; 3: United Lincolnshire Hospitals NHS Trust, Lincoln, UK

American Society of Clinical Oncology (ASCO) Annual Meeting 2015.

GATA-1, FOG-1, and FLI-1 regulation in essential thrombocythemia independently from JAK2 and CALR mutations.

C.R. Rinaldi[1,2], James Lally[1], L Brown[1], Ciaren Graham[1];

1: School of Life Sciences, University of Lincoln, Lincoln UK; 2: United Lincolnshire Hospitals NHS Trust, Lincoln, UK

British Society of Haematology (BSH) Annual Meeting 2015

Expression of the transcription factors FOG1, FLI-1 and GATA-1 in the peripheral blood of essential thrombocythaemia patients.

J Lally[1], L Brown[1], C Graham[1] and CR Rinaldi[1,2]

1: University of Lincoln, School of Life Sciences. 2: United Lincolnshire NHS Hospitals Trust

9.2: References

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